EXHIBIT 10

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U.S. PATENT: *9,708,361*

ISSUE DATE: *July 18, 2017*

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T. WALLACE

Certifying Officer

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(12) **United States Patent**
Watanabe et al.

(10) **Patent No.: US 9,708,361 B2**
(45) **Date of Patent: Jul. 18, 2017**

(54) **ANTISENSE NUCLEIC ACIDS**

(71) Applicants: **NIPPON SHINYAKU CO., LTD.**, Kyoto-shi, Kyoto (JP); **NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY**, Kodaira-shi, Tokyo (JP)

(72) Inventors: **Naoki Watanabe**, Tsukuba (JP); **Youhei Satou**, Tsukuba (JP); **Shin'ichi Takeda**, Kodaira (JP); **Tetsuya Nagata**, Kodaira (JP)

(73) Assignees: **NIPPON SHINYAKU CO., LTD.**, Kyoto-shi, Kyoto (JP); **NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY**, Tokyo (JP)

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(22) Filed: **Feb. 6, 2015**

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Related U.S. Application Data

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(30) **Foreign Application Priority Data**

Sep. 1, 2010 (JP) 2010-196032

(51) **Int. Cl.**

C07H 21/02	(2006.01)
C07H 21/04	(2006.01)
A61K 31/70	(2006.01)
C12N 15/11	(2006.01)
C12N 15/113	(2010.01)
C07H 21/00	(2006.01)
C12N 5/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C07H 21/04*** (2013.01); ***C07H 21/00*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 15/113*** (2013.01); *C12N 2310/11* (2013.01); *C12N 2310/315* (2013.01); *C12N 2310/3145* (2013.01); *C12N 2310/321* (2013.01); *C12N 2310/3525* (2013.01); *C12N 2320/33* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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Primary Examiner — Sean McGarry
(74) *Attorney, Agent, or Firm* — Drinker Biddle & Reath LLP

(57) **ABSTRACT**

The present invention provides an oligomer which efficiently enables to cause skipping of the 53rd exon in the human dystrophin gene. Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.

7 Claims, 19 Drawing Sheets

Figure 1

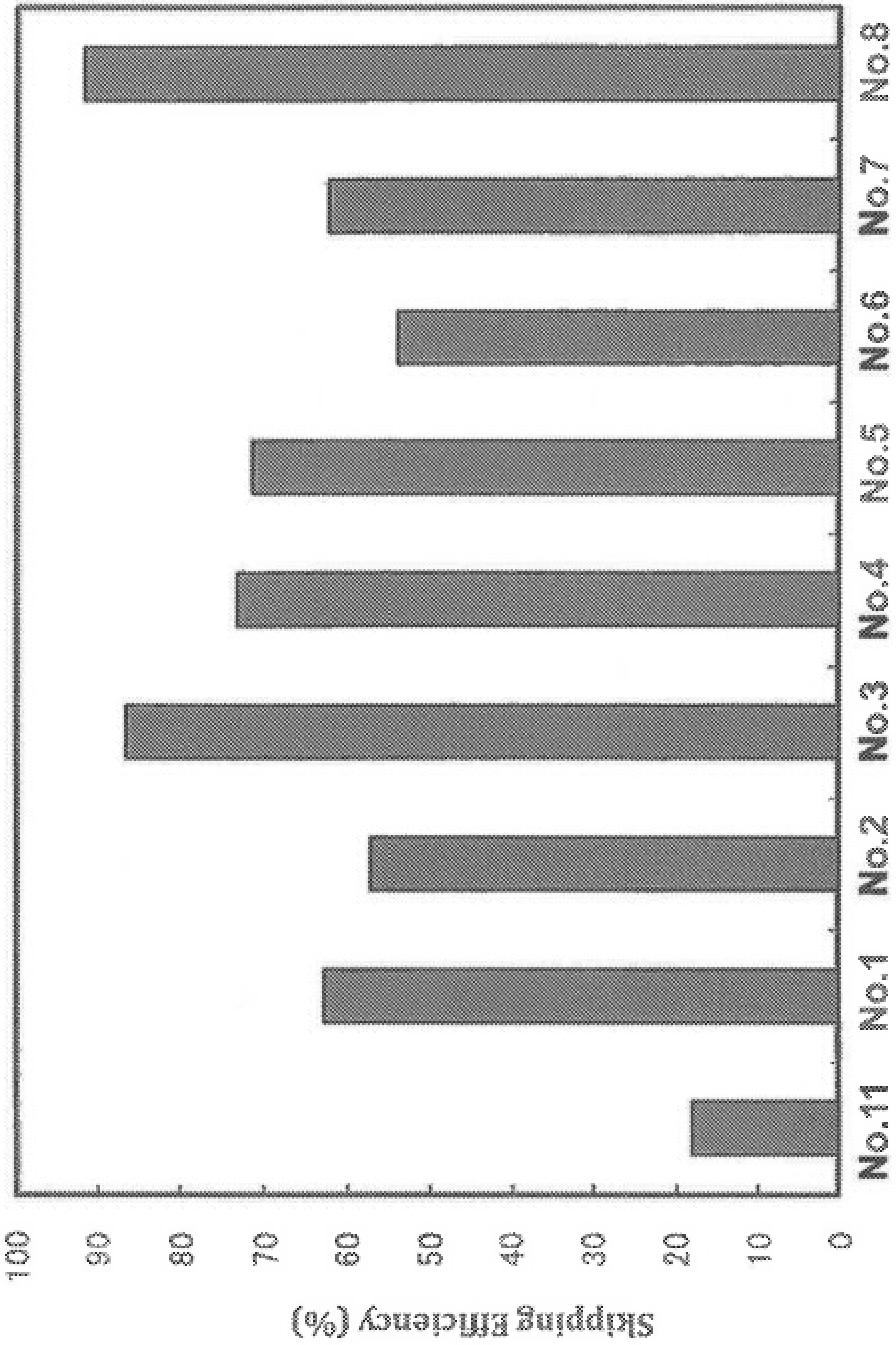

Skipping Efficiency (%)
100
90
80
70
60
50
40
30
20
10
0
No.11
No.1
No.2
No.3
No.4
No.5
No.6
No.7
No.8

Figure 2

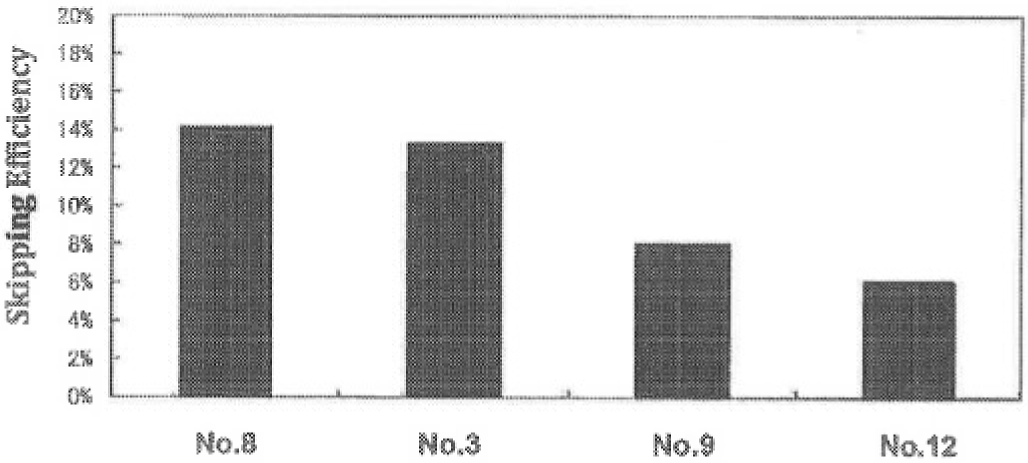
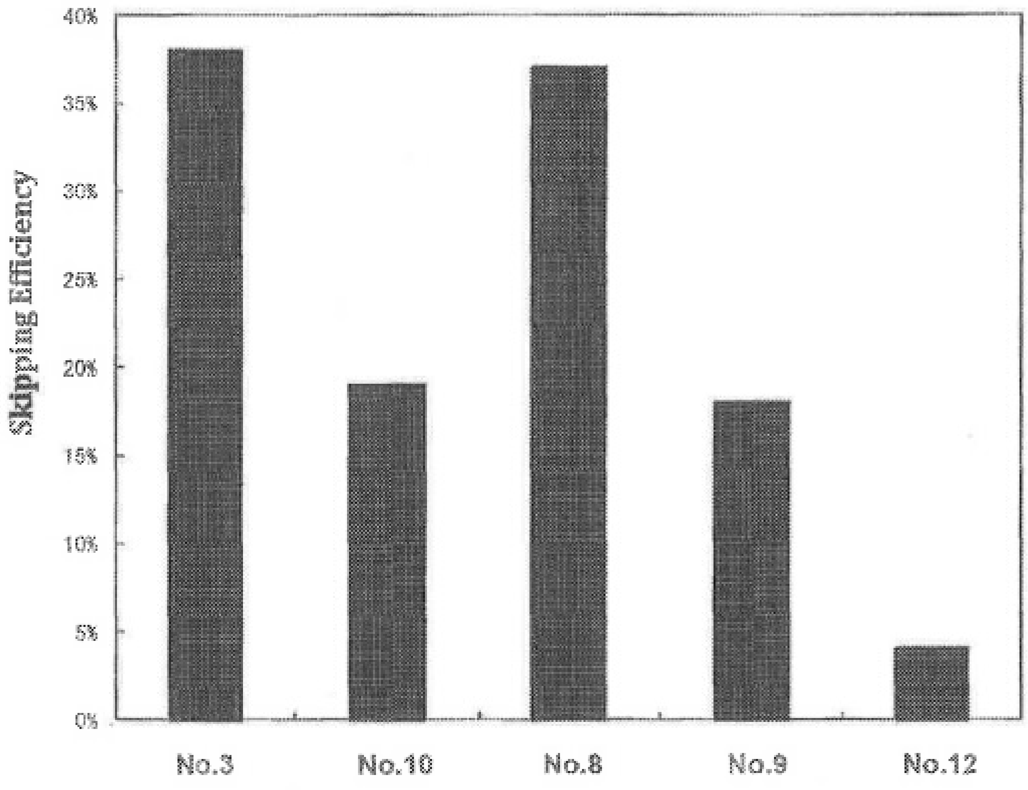


Figure 3



40%
35%
30%
25%
20%
15%
10%
5%
0%
Skipping Efficiency
No.3
No.10
No.8
No.9
No.12

Figure 4

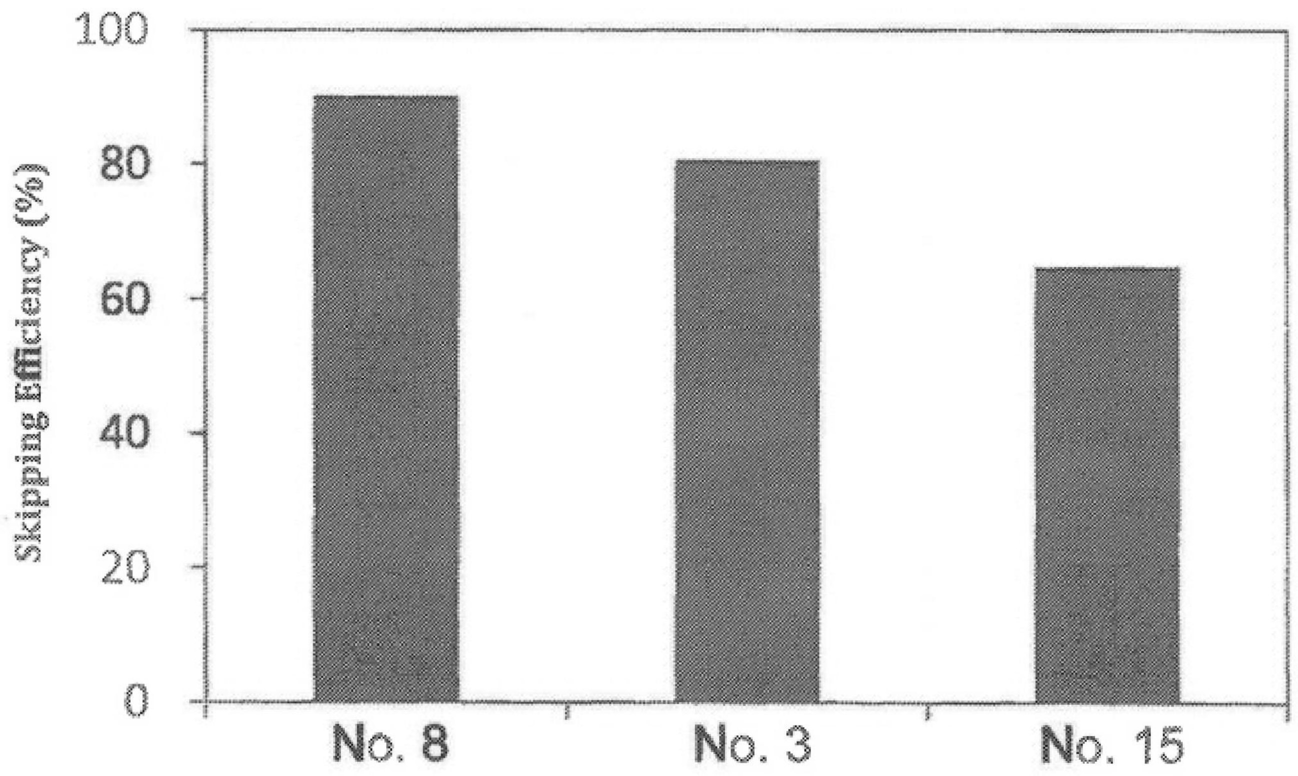


Figure 5

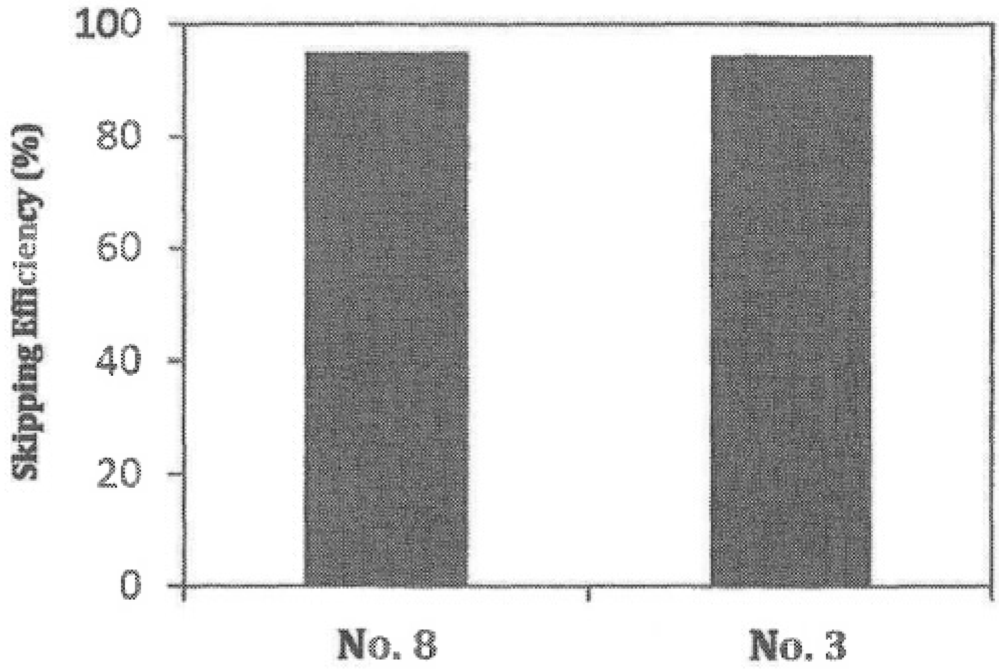


Figure 6

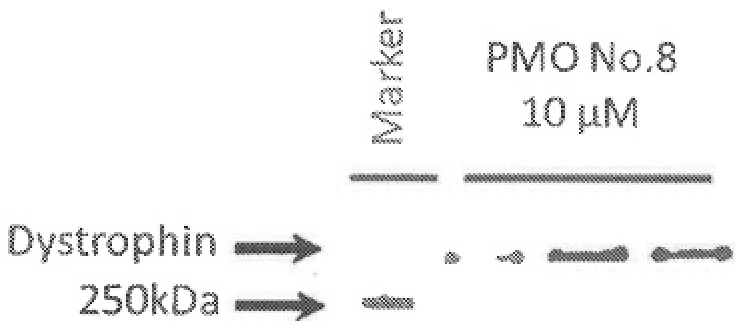
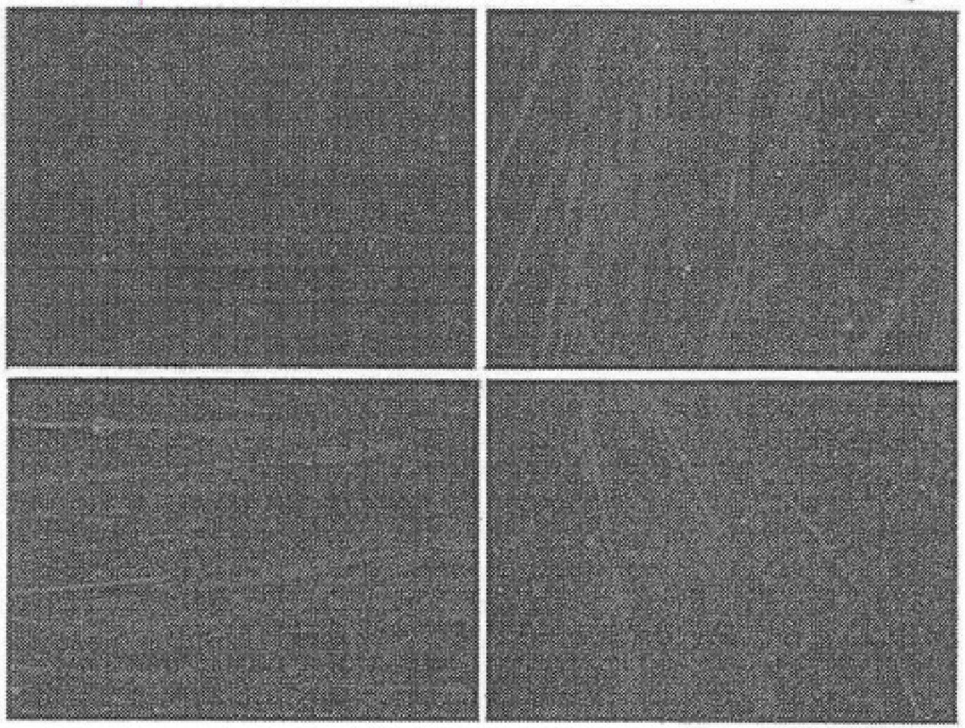


Figure 7

Patient with Exon 48-52 Deletion (No PMO)

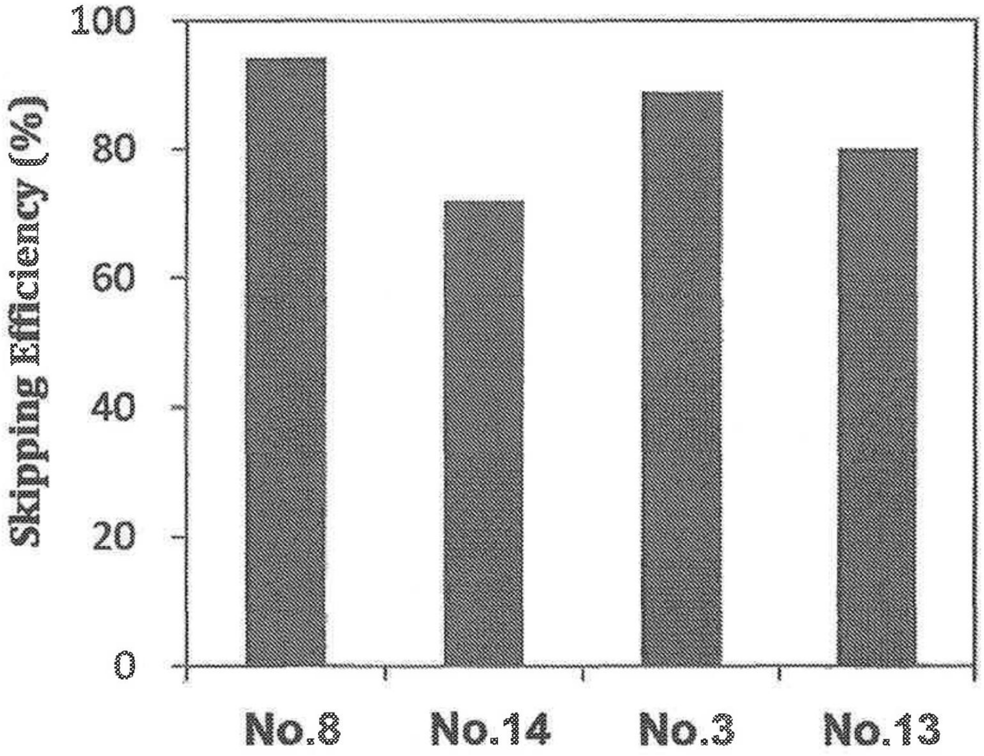
Patient with Exon 45-52 Deletion (PMO No. 8)

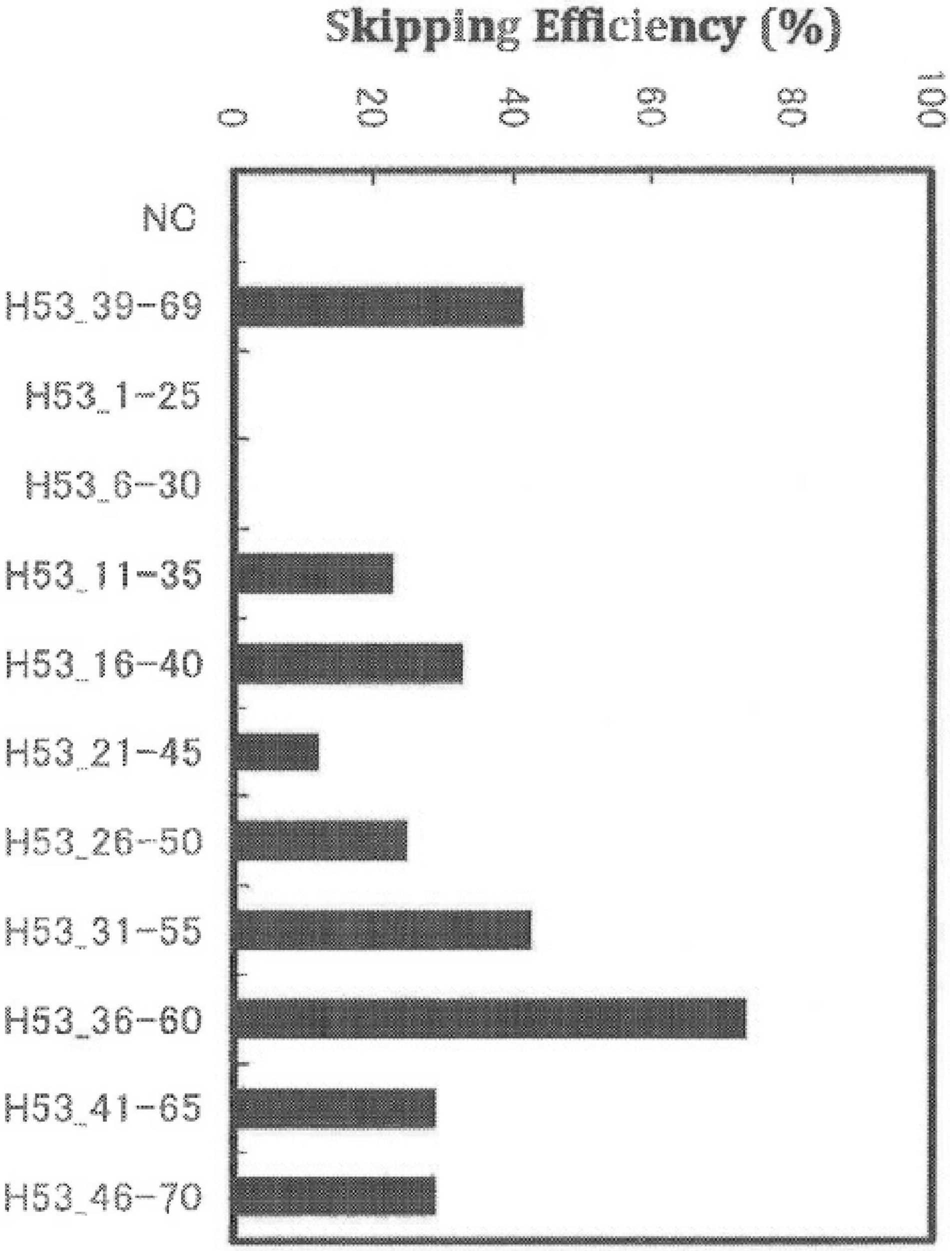


Patient with Exon 48-52 Deletion (PMO No. 8)

Patient with Exon 48-52 Deletion (PMO No. 3)

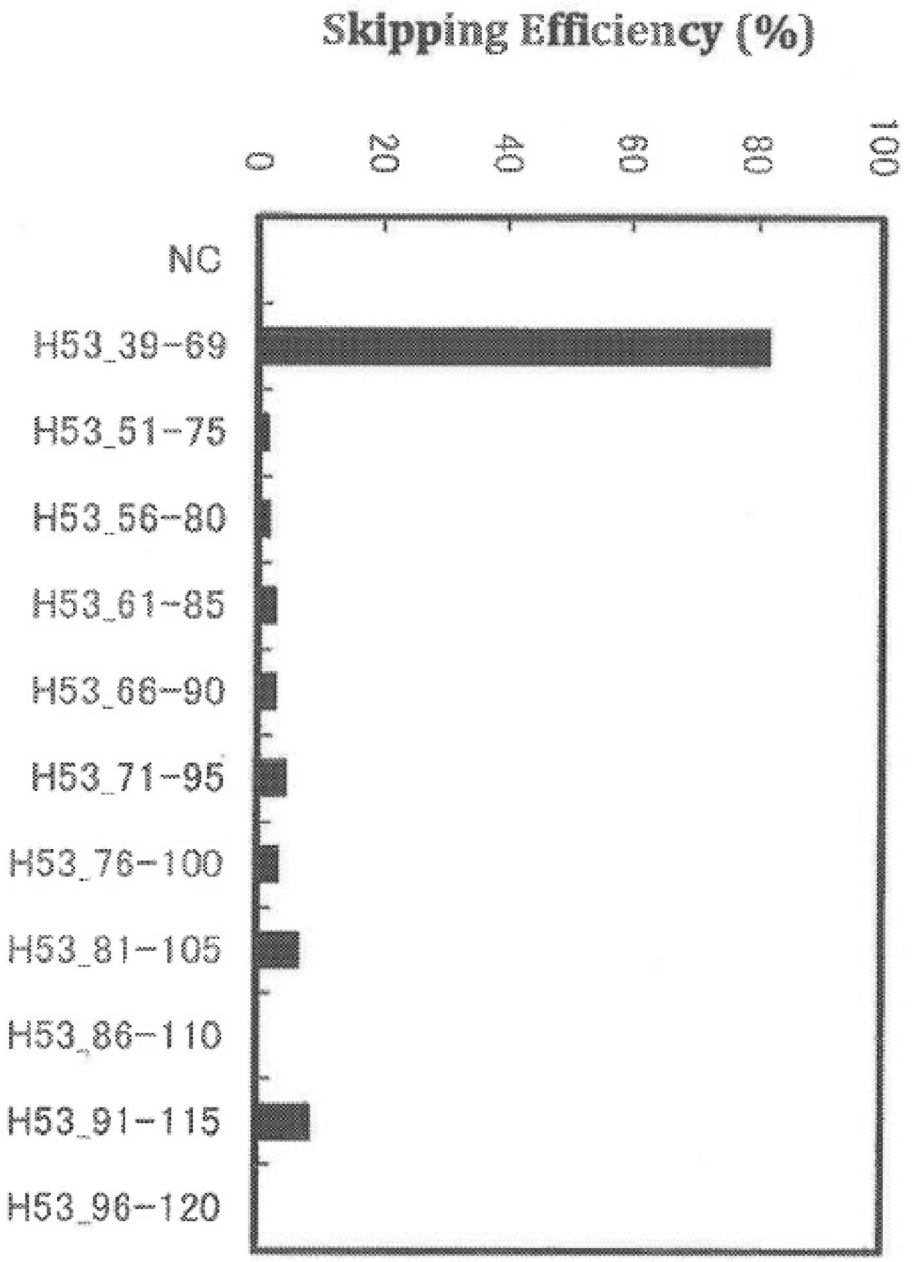
Figure 8





Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_1-25
H53_6-30
H53_11-35
H53_16-40
H53_21-45
H53_26-50
H53_31-55
H53_36-60
H53_41-65
H53_46-70

Figure 9



Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_51-75
H53_56-80
H53_61-85
H53_66-90
H53_71-95
H53_76-100
H53_81-105
H53_86-110
H53_91-115
H53_96-120

Figure 10

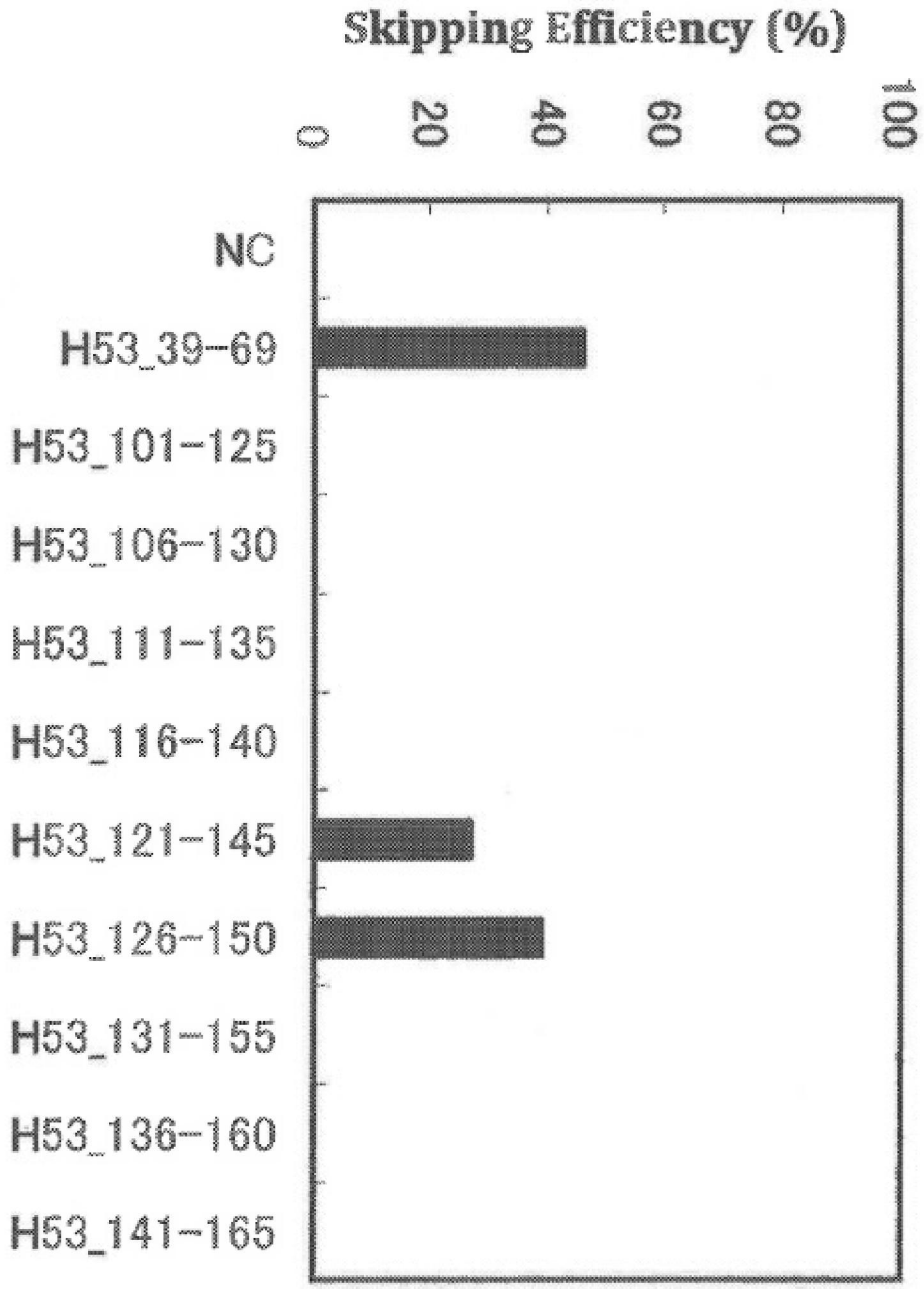

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_101-125
H53_106-130
H53_111-135
H53_116-140
H53_121-145
H53_126-150
H53_131-155
H53_136-160
H53_141-165

Figure 11

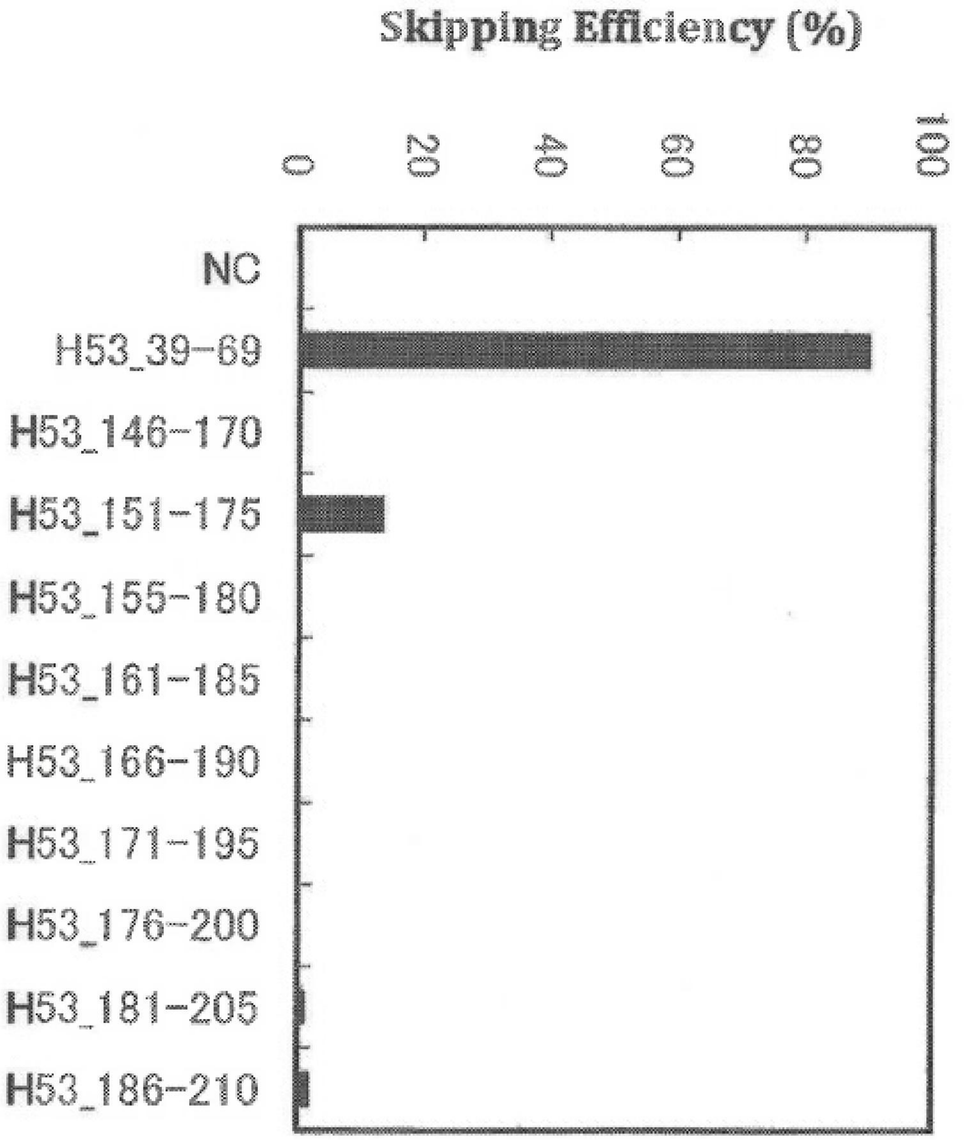

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_146-170
H53_151-175
H53_155-180
H53_161-185
H53_166-190
H53_171-195
H53_176-200
H53_181-205
H53_186-210

Figure 12

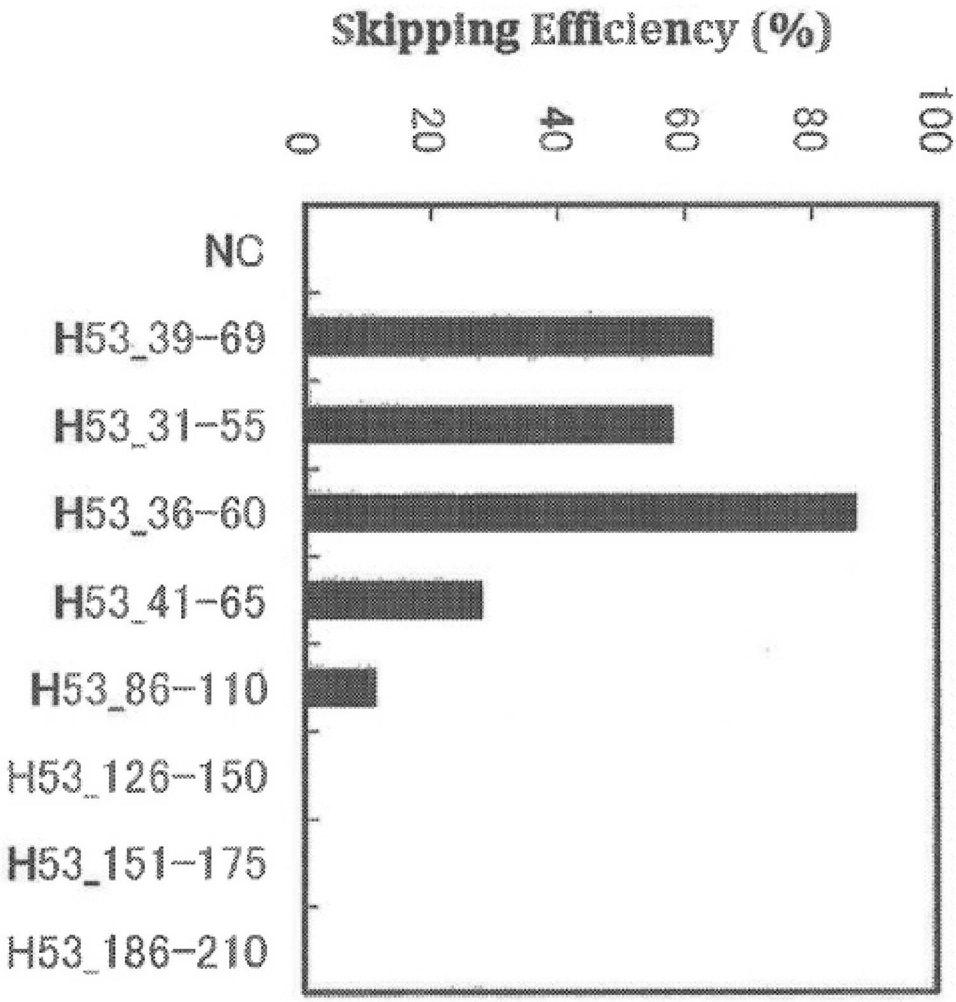

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_31-55
H53_36-60
H53_41-65
H53_86-110
H53_126-150
H53_151-175
H53_186-210

Figure 13

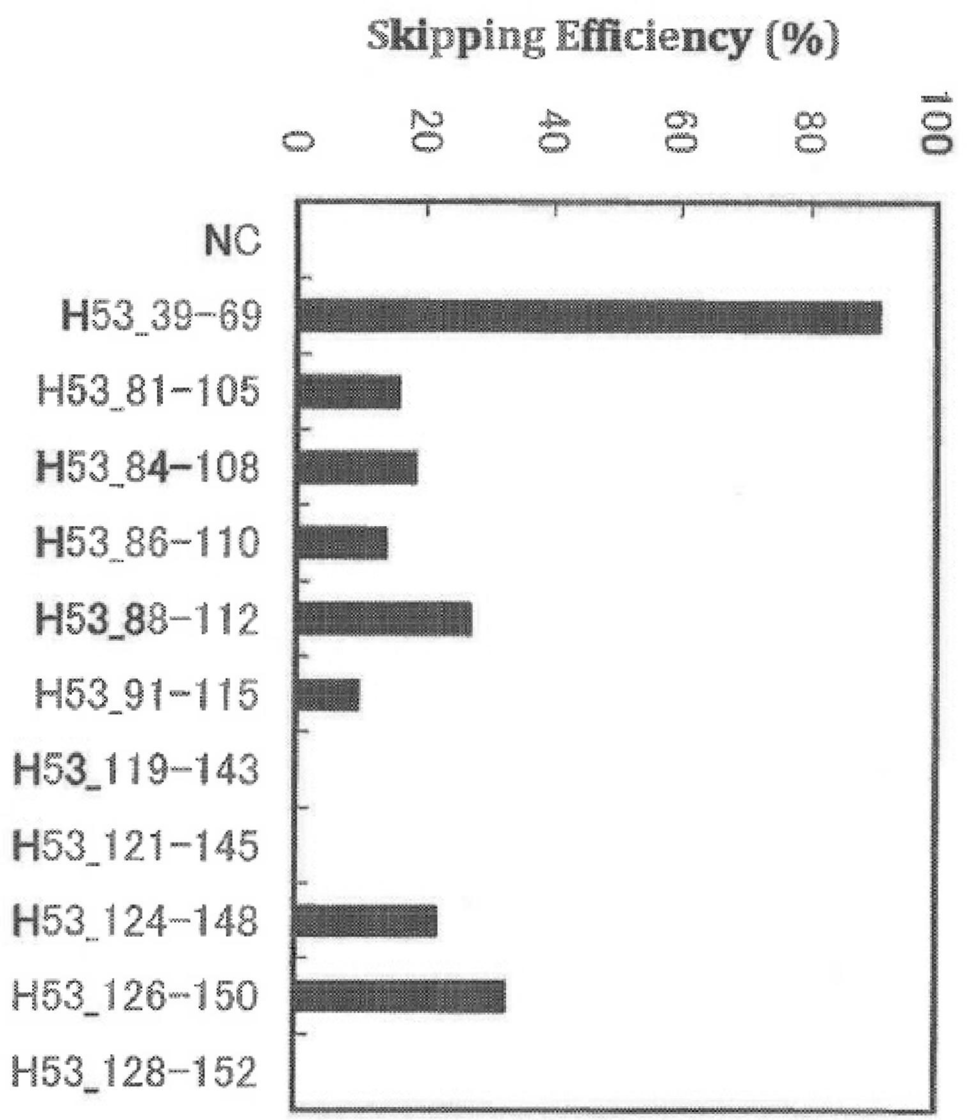

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_81-105
H53_84-108
H53_86-110
H53_88-112
H53_91-115
H53_119-143
H53_121-145
H53_124-148
H53_126-150
H53_128-152

Figure 14

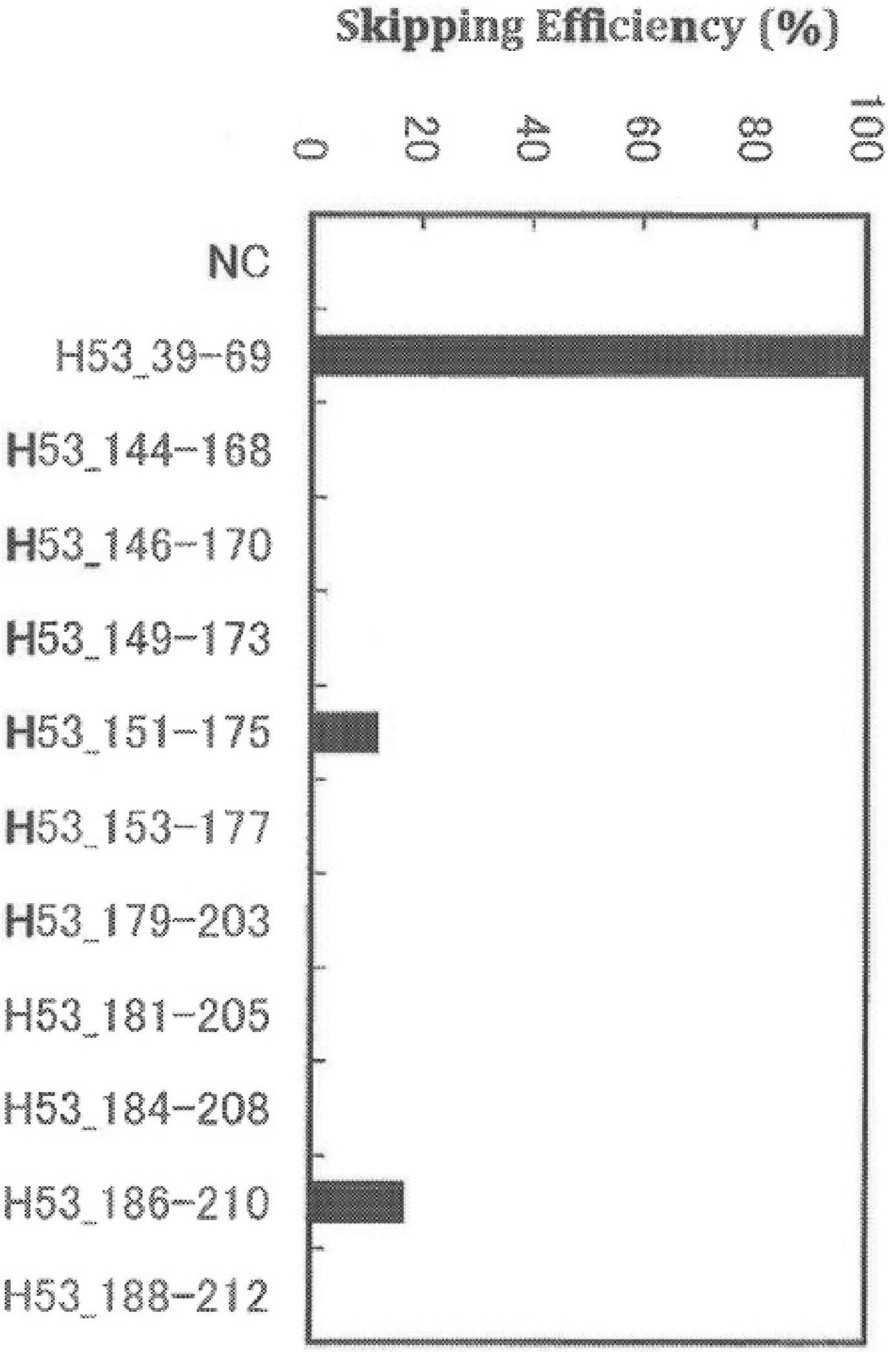

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_144-168
H53_146-170
H53_149-173
H53_151-175
H53_153-177
H53_179-203
H53_181-205
H53_184-208
H53_186-210
H53_188-212

Figure 15

Figure 16

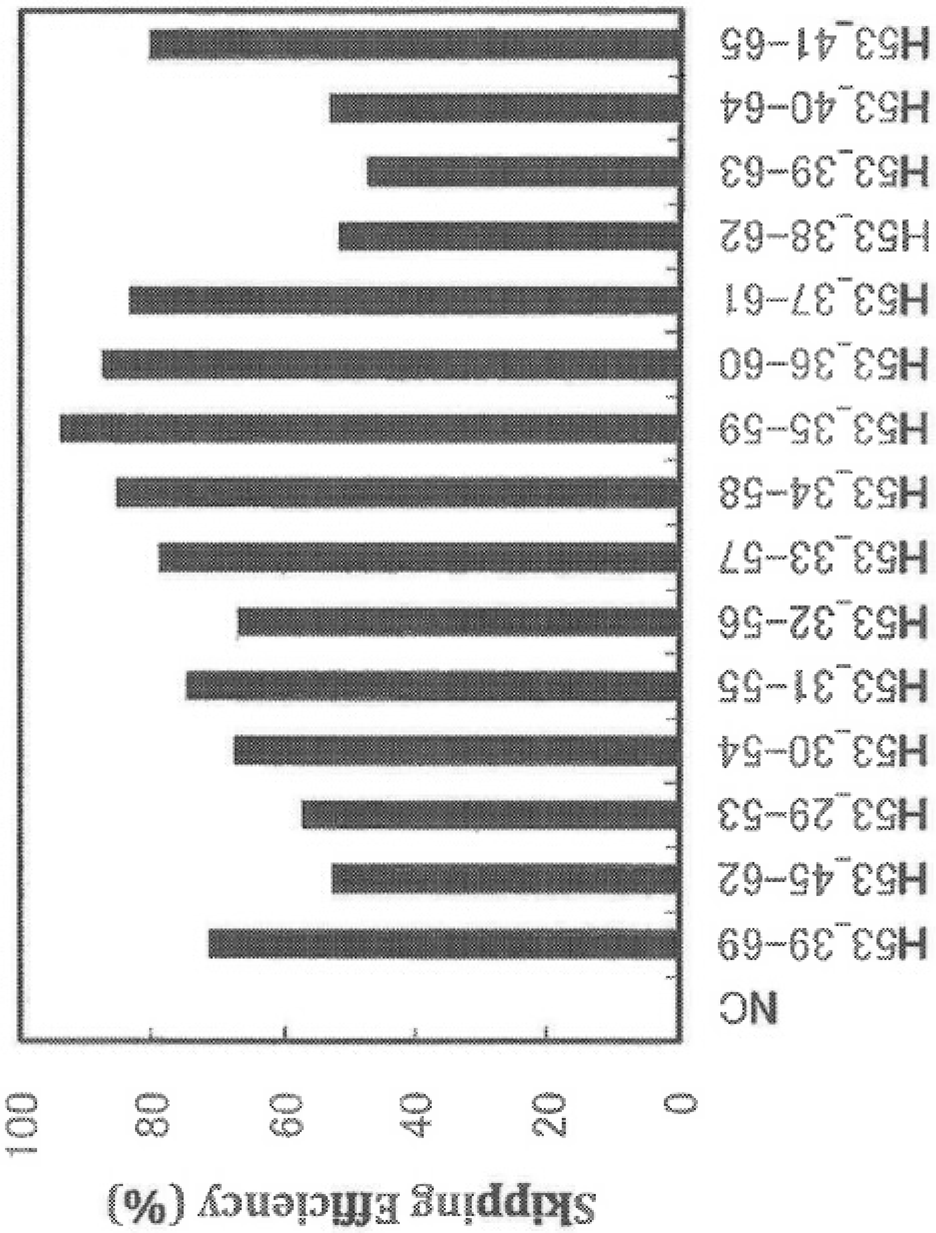
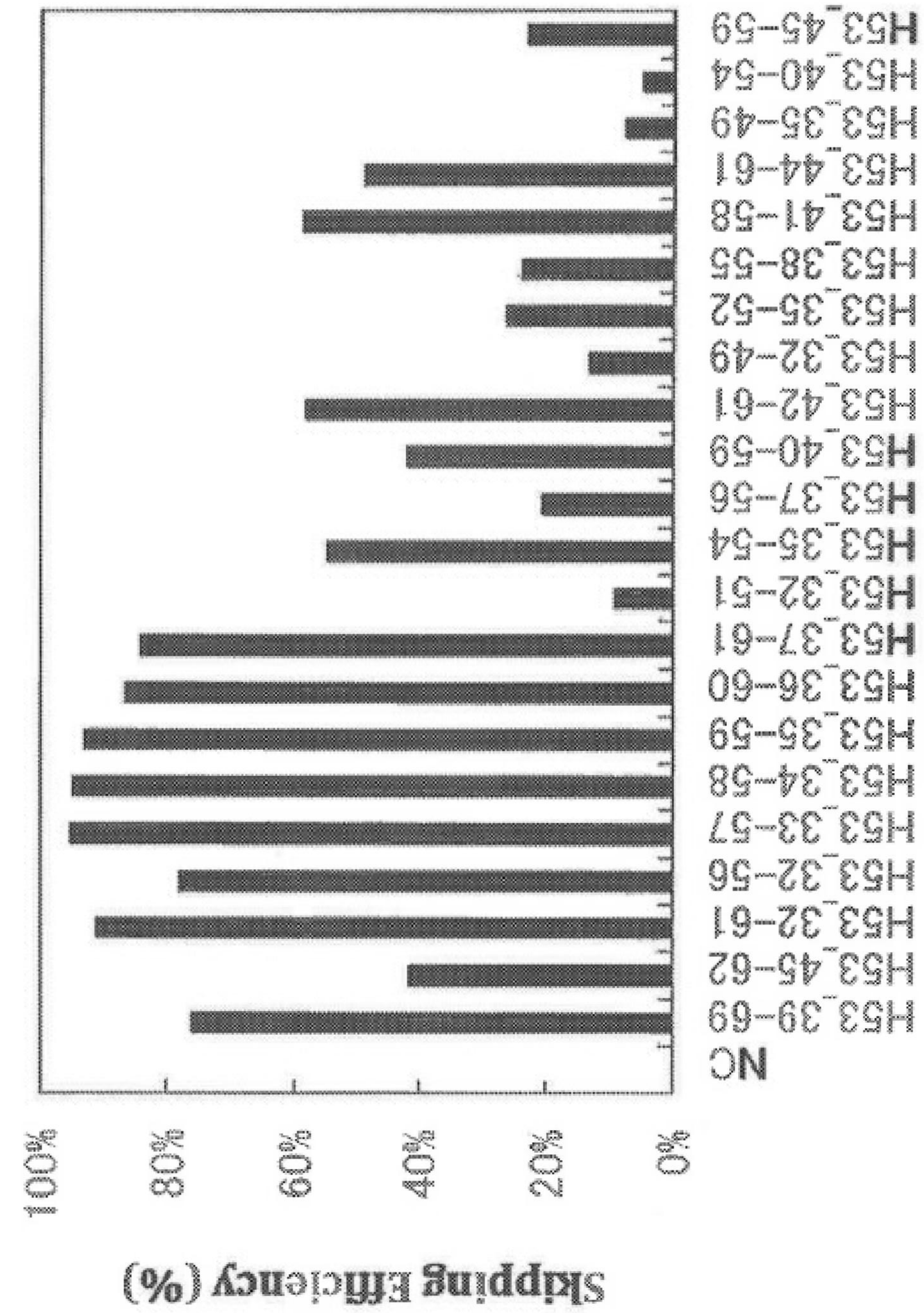

H53_41-65
H53_40-64
H53_39-63
H53_38-62
H53_37-61
H53_36-60
H53_35-59
H53_34-58
H53_33-57
H53_32-56
H53_31-55
H53_30-54
H53_29-53
H53_45-62
H53_39-69
NC
100
80
60
40
20
0
Skipping Efficiency (%)

Figure 17


Skipping Efficiency (%)
100%
80%
60%
40%
20%
0%
NC
H53_39-69
H53_45-62
H53_32-61
H53_32-56
H53_33-57
H53_34-58
H53_35-59
H53_36-60
H53_37-61
H53_32-51
H53_35-54
H53_37-56
H53_40-59
H53_42-61
H53_32-49
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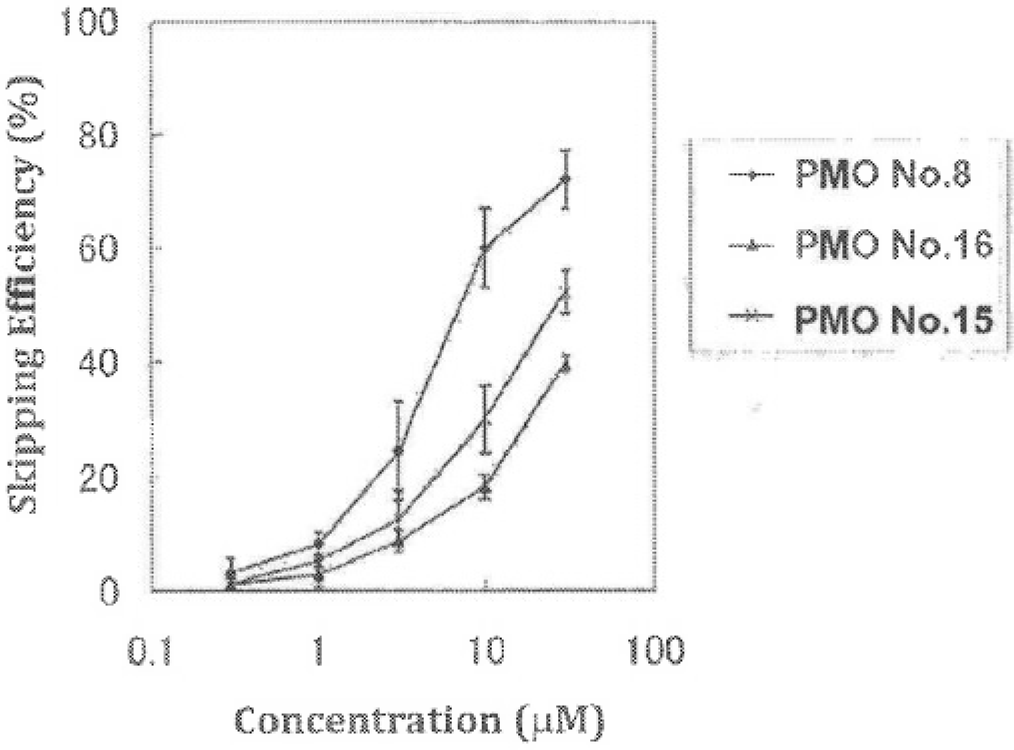
H53_41-58
H53_44-61
H53_35-49
H53_40-54
H53_45-59



100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.16
PMO No.15

Figure 18

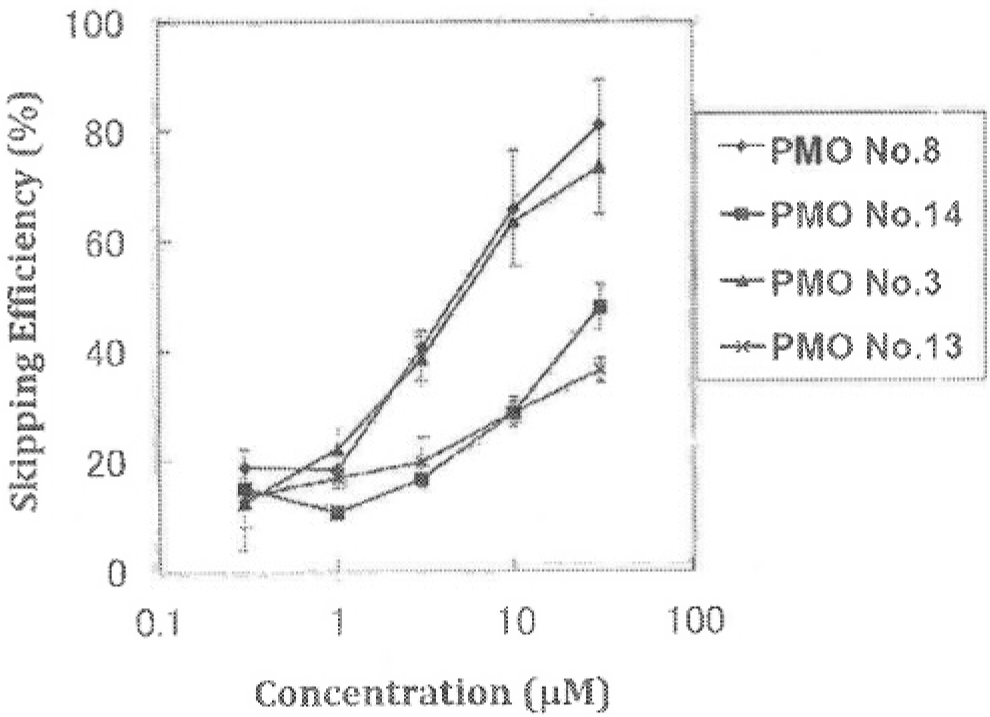

100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.14
PMO No.3
PMO No.13

Figure 19

ANTISENSE NUCLEIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS

This is a Continuation of copending application Ser. No. 13/819,520, filed Apr. 10, 2013, which is a PCT National Stage of PCT/JP2011/070318 filed Aug. 31, 2011, which claims priority to JP Application No, 2010-196032 filed Sep. 1, 2010.

SEQUENCE LISTING

A Sequence Listing containing SEQ ID NO: 1-123 is incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to an antisense oligomer which causes skipping of exon 53 in the human dystrophin gene, and a pharmaceutical composition comprising the oligomer.

BACKGROUND ART

Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscular dystrophy that affects one in about 3,500 newborn boys. Although the motor functions are rarely different from healthy humans in infancy and childhood, muscle weakness is observed in children from around 4 to 5 years old. Then, muscle weakness progresses to the loss of ambulation by about 12 years old and death due to cardiac or respiratory insufficiency in the twenties. DMD is such a severe disorder. At present, there is no effective therapy for DMD available, and it has been strongly desired to develop a novel therapeutic agent.

DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on X chromosome and is a huge gene consisting of 2.2 million DNA nucleotide pairs. DNA is transcribed into mRNA precursors, and introns are removed by splicing to synthesize mRNA in which 79 exons are joined together. This mRNA is translated into 3,685 amino acids to produce the dystrophin protein. The dystrophin protein is associated with the maintenance of membrane stability in muscle cells and necessary to make muscle cells less fragile. The dystrophin gene from patients with DMD contains a mutation and hence, the dystrophin protein, which is functional in muscle cells, is rarely expressed. Therefore, the structure of muscle cells cannot be maintained in the body of the patients with DMD, leading to a large influx of calcium ions into muscle cells. Consequently, an inflammation-like response occurs to promote fibrosis so that muscle cells can be regenerated only with difficulty.

Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene. The symptoms involve muscle weakness accompanied by atrophy of muscle but are typically mild and slow in the progress of muscle weakness, when compared to DMD. In many cases, its onset is in adulthood. Differences in clinical symptoms between DMD and BMD are considered to reside in whether the reading frame for amino acids on the translation of dystrophin mRNA into the dystrophin protein is disrupted by the mutation or not (Non-Patent Document 1). More specifically, in DMD, the presence of mutation shifts the amino acid reading frame so that the expression of functional dystrophin protein is abolished, whereas in BMD the dystrophin protein that functions, though imperfectly, is produced because the amino acid reading frame is preserved, while a part of the exons are deleted by the mutation.

Exon skipping is expected to serve as a method for treating DMD. This method involves modifying splicing to restore the amino acid reading frame of dystrophin mRNA and induce expression of the dystrophin protein having the function partially restored (Non-Patent Document 2). The amino acid sequence part, which is a target for exon skipping, will be lost. For this reason, the dystrophin protein expressed by this treatment becomes shorter than normal one but since the amino acid reading frame is maintained, the function to stabilize muscle cells is partially retained. Consequently, it is expected that exon skipping will lead DMD to the similar symptoms to that of BMD which is milder. The exon skipping approach has passed the animal tests using mice or dogs and now is currently assessed in clinical trials on human DMD patients.

The skipping of an exon can be induced by binding of antisense nucleic acids targeting either 5' or 3' splice site or both sites, or exon-internal sites. An exon will only be included in the mRNA when both splice sites thereof are recognized by the spliceosome complex. Thus, exon skipping can be induced by targeting the splice sites with antisense nucleic acids. Furthermore, the binding of an SR protein to an exonic splicing enhancer (ESE) is considered necessary for an exon to be recognized by the splicing mechanism. Accordingly, exon skipping can also be induced by targeting ESE.

Since a mutation of the dystrophin gene may vary depending on DMD patients, antisense nucleic acids need to be desined based on the site or type of respective genetic mutation. In the past, antisense nucleic acids that induce exon skipping for all 79 exons were produced by Steve Wilton, et al., University of Western Australia (Non-Patent Document 3), and the antisense nucleic acids which induce exon skipping for 39 exons were produced by Annemieke Aartsma-Rus, et al., Netherlands (Non-Patent Document 4).

It is considered that approximately 8% of all DMD patients may be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, a plurality of research organizations reported on the studies where exon 53 in the dystrophin gene was targeted for exon skipping (Patent Documents 1 to 4; Non-Patent Document 5). However, a technique for skipping exon 53 with a high efficiency has not yet been established.

Patent Document 1: International Publication WO 2006/000057

Patent Document 2: International Publication WO 2004/048570

Patent Document 3: US 2010/0168212

Patent Document 4: International Publication WO 2010/048586

Non-Patent Document 1: Monaco A. P. et al., Genomics 1988; 2: p. 90-95

Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p. 167-172

Non-Patent Document 3: Wilton S. D., et al., Molecular Therapy 2007: 15: p. 1288-96

Non-Patent Document 4: Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77

Non-Patent Document 5: Linda J. Popplewell et al., (2010) Neuromuscular Disorders, vol. 20, no. 2, p. 102-10

DISCLOSURE OF THE INVENTION

Under the foregoing circumstances, antisense oligomers that strongly induce exon 53 skipping in the dystrophin gene and muscular dystrophy therapeutics comprising oligomers thereof have been desired.

As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

That is, the present invention is as follows.

[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 31st to the 53rd, the 31 st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[2] The antisense oligomer according to [1] above, which is an oligonucleotide.

[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SiH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

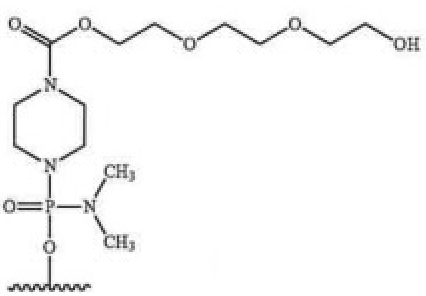
[5] The antisense oligomer according to [3] or [4] above, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

[6] The antisense oligomer according to [1] above, which is a morpholino oligomer.

[7] The antisense oligomer according to [6] above, which is a phosphorodiamidate morpholino oligomer.

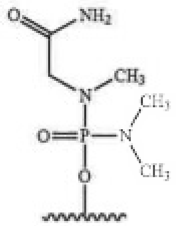
[8] The antisense oligomer according to any one of [1] to [7] above, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:

(1)

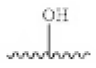


-continued

(2)



(3)



[9] The antisense oligomer according to any one of [1] to [8] above, consisting of a nucleotide sequence complementary to the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

[10] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 2 to 37.

[11] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 11, 17, 23, 29 and 35.

[12] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by SEQ ID NO: 11 or 35.

[13] A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to any one of [1] to [12] above, or a pharmaceutically acceptable salt or hydrate thereof.

The antisense oligomer of the present invention can induce exon 53 skipping in the human dystrophin gene with a high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering the pharmaceutical composition of the present invention.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

FIG. 2 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human normal tissue-derived fibroblasts (TIG-119 cells) to induce differentiation into muscle cells.

FIG. 3 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human DMD patient-derived fibroblasts (5017 cells) to induce differentiation into muscle cells.

FIG. 4 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. 5 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 6 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 7 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 8 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. 9 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 10 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 11 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 12 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 13 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 14 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 15 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 16 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 17 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 18 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

FIG. 19 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

All of the publications, published patent applications, patents and other patent documents cited in the specification are herein incorporated by reference in their entirety. The specification hereby incorporates by reference the contents of the specification and drawings in the Japanese Patent Application (No. 2010-196032) filed Sep. 1, 2010, from which the priority was claimed.

1. Antisense Oligomer

The present invention provides the antisense oligomer (hereinafter referred to as the "oligomer of the present invention") which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of the 31 st to the 53rd, the 31 st to the 54th, the 31 st to the 55th, the 31 st to the 56th, the 31 st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[Exon 53 in Human Dystrophin Gene]

In the present invention, the term "gene" is intended to mean a genomic gene and also include cDNA, mRNA precursor and mRNA. Preferably, the gene is mRNA precursor, i.e., pre-mRNA.

In the human genome, the human dystrophin gene locates at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest gene among known human genes. However, the coding regions of the human dystrophin gene are only 14 kb, distributed as 79 exons throughout the human dystrophin gene (Roberts, R G., et al., Genomics, 16: 536-538 (1993)). The pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to generate mature mRNA of 14 kb. The nucleotide sequence of human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is represented by SEQ ID NO: 1.

The oligomer of the present invention is designed to cause skipping of exon 53 in the human dystrophin gene, thereby modifying the protein encoded by DMD type of dystrophin gene into the BMD type of dystrophin protein. Accordingly, exon 53 in the dystrophin gene that is the target of exon skipping by the oligomer of the present invention includes both wild and mutant types.

Specifically, exon 53 mutants of the human dystrophin gene include the polynucleotides defined in (a) or (b) below.

(a) A polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1; and,

(b) A polynucleotide consisting of a nucleotide sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO: 1.

As used herein, the term "polynucleotide" is intended to mean DNA or RNA.

As used herein, the term "polynucleotide that hybridizes under stringent conditions" refers to, for example, a polynucleotide obtained by colony hybridization, plaque hybridization, Southern hybridization or the like, using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of, e.g., SEQ ID NO: 1. The hybridization method which may be used includes methods described in, for example, "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001,"

"Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does not only refers to a nucleotide sequence 100% complementary to the target nucleotide sequence but also refers to a complementary nucleotide sequence that may contain, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence.

As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions or high stringent conditions. The term "low stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 32° C. The term "moderate stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 42° C., or 5×SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42° C. The term "high stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 50° C. or 0.2×SSC, 0.1% SDS at 65° C. Under these conditions, polynucleotides with higher homology are expected to be obtained efficiently at higher temperatures, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and those skilled in the art may appropriately select these factors to achieve similar stringency.

When commercially available kits are used for hybridization, for example, an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55° C., thereby detecting hybridized polynucleotides. Alternatively, in producing a probe based on the entire or part of the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (e.g., a PCR Labeling Mix (Roche Diagnostics), etc.).

In addition to the polynucleotides described above, other polynucleotides that can be hybridized include polynucleotides having 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST using the default parameters.

The identity between nucleotide sequences may be determined using algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). Programs called BLASTN and BLASTX based on the BLAST algorithm have been developed (Altschul S F, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score=100 and wordlength=12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are employed.

Examples of the nucleotide sequences complementary to the sequences consisting of the 31 st to the 53rd, the 31 st to the 54th, the 31 st to the 55th, the 31 st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th and the 36th to the 58th nucleotides, from the 5' end of exon 53.

TABLE 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31-53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31-54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3
31-55	5'-CTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 4
31-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31-57	3'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO; 6
31-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32-53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8
32-54	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9
32-55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 10
32-56	5'CCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 11
32-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 12

TABLE 1-continued

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
32-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 13
33-53	5'-CCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 14
33-54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 15
33-55	5'-CTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 16
33-56	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 17
33-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 18
33-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 19
34-53	5'-CCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 20
34-54	5'TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34-55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34-56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34-57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35-53	5'-CCGGTTCTGAAGGTGTTCT-'3	SEQ ID NO: 26
35-54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35-55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28
35-56	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35-57	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35-58	5'TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36-53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36-54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36-55	5'-CTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 34
36-56	5'-CCTCCGGTTCTCGAAGGTGTTC-3'	SEQ ID NO: 35
36-57	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36-58	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 37

It is preferred that the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th, the 33rd to the 56th, the 34th to the 56th, the 35th to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23, SEQ ID NO: 29 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

Preferably, the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

The term "cause skipping of the 53rd exon in the human dystrophin gene" is intended to mean that by binding of the oligomer of the present invention to the site corresponding to exon 53 of the transcript (e.g., pre-mRNA) of the human dystrophin gene, for example, the nucleotide sequence corresponding to the 5' end of exon 54 is spliced at the 3' side of the nucleotide sequence corresponding to the 3' end of exon 51 in DMD patients with deletion of, exon 52 when the transcript undergoes splicing, thus resulting in formation of mature mRNA which is free of codon frame shift.

Accordingly, it is not required for the oligomer of the present invention to have a nucleotide sequence 100% complementary to the target sequence, as far as it causes exon 53 skipping in the human dystrophin gene. The oligomer of the present invention may include, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target sequence.

Herein, the term "binding" described above is intended to mean that when the oligomer of the present invention is mixed with the transcript of human dystrophin gene, both are hybridized under physiological conditions to form a double strand nucleic acid. The term "under physiological conditions" refers to conditions set to mimic the in vivo environment in terms of pH, salt composition and tempera-

ture. The conditions are, for example, 25 to 40° C., preferably 37° C., pH 5 to 8, preferably pH 7.4 and 150 mM of sodium chloride concentration.

Whether the skipping of exon 53 in the human dystrophin gene is caused or not can be confirmed by introducing the oligomer of the present invention into a dystrophin expression cell (e.g., human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human dystrophin gene from the total RNA of the dystrophin expression cell by RT-PCR and performing nested PCR or sequence analysis on the PCR amplified product.

The skipping efficiency can be determined as follows. The mRNA for the human dystrophin gene is collected from test cells; in the mRNA, the polynucleotide level "A" of the band where exon 53 is skipped and the polynucleotide level "B" of the band where exon 53 is not skipped are measured. Using these measurement values of "A" and "B," the efficiency is calculated by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

The oligomer of the present invention includes, for example, an oligonucleotide, morpholino oligomer or peptide nucleic acid (PNA), having a length of 18 to 28 nucleotides. The length is preferably from 21 to 25 nucleotides and morpholino oligomers are preferred.

The oligonucleotide described above (hereinafter referred to as "the oligonucleotide of the present invention") is the oligomer of the present invention composed of nucleotides as constituent units. Such nucleotides may be any of ribonucleotides, deoxyribonucleotides and modified nucleotides.

The modified nucleotide refers to one having fully or partly modified nucleobases, sugar moieties and/or phosphate-binding regions, which constitute the ribonucleotide or deoxyribonucleotide.

The nucleobase includes, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil, and modified bases thereof. Examples of such modified nucleobases include, but not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines (e.g., 5-methylcytosine), 5-alkyluracils (e.g., 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methylguanine, 5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole, xanthine, etc.

Modification of the sugar moiety may include, for example, modifications at the 2'-position of ribose and modifications of the other positions of the sugar. The modification at the 2'-position of ribose includes replacement of the 2'-OH of ribose with OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or I, wherein R represents an alkyl or an aryl and R' represents an alkylene.

The modification for the other positions of the sugar includes, for example, replacement of O at the 4' position of ribose or deoxyribose with S, bridging between 2' and 4' positions of the sugar, e.g., LNA (locked nucleic acid) or ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but is not limited thereto.

A modification of the phosphate-binding region includes, for example, a modification of replacing phosphodiester bond with phosphorothioate bond, phosphorodithioate bond, alkyl phosphonate bond, phosphoroamidate bond or boranophosphate bond (Enya et al: Bioorganic & Medicinal Chemistry, 2008, 18, 9154-9160) (cf., e.g., Japan Domestic Re-Publications of PCT Application Nos. 2006/129594 and 2006/038608).

The alkyl is preferably a straight or branched alkyl having 1 to 6 carbon atoms. Specific examples include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, tert-pentyl, n-hexyl and isohexyl. The alkyl may optionally be substituted. Examples of such substituents are a halogen, an alkoxy, cyano and nitro. The alkyl may be substituted with 1 to 3 substituents.

The cycloalkyl is preferably a cycloalkyl having 5 to 12 carbon atoms. Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

The halogen includes fluorine, chlorine, bromine and iodine.

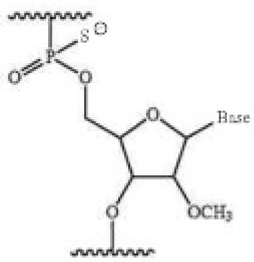
The alkoxy is a straight or branched alkoxy having 1 to 6 carbon atoms such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy, tert-butoxy, n-pentyloxy, isopentyloxy, n-hexyloxy, isohexyloxy, etc. Among others, an alkoxy having 1 to 3 carbon atoms is preferred.

The aryl is preferably an aryl having 6 to 10 carbon atoms. Specific examples include phenyl, α-naphthyl and β-naphthyl. Among others, phenyl is preferred. The aryl may optionally be substituted. Examples of such substituents are an alkyl, a halogen, an alkoxy, cyano and nitro. The aryl may be substituted with one to three of such substituents.

The alkylene is preferably a straight or branched alkylene having 1 to 6 carbon atoms. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl)trimethylene and 1-(methyl)tetramethylene.

The acyl includes a straight or branched alkanoyl or aroyl. Examples of the alkanoyl include formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl, hexanoyl, etc. Examples of the aroyl include benzoyl, toluoyl and naphthoyl. The aroyl may optionally be substituted at substitutable positions and may be substituted with an alkyl(s).

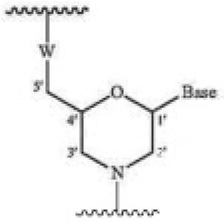
Preferably, the oligonucleotide of the present invention is the oligomer of the present invention containing a constituent unit represented by general formula below wherein the —OH group at position 2' of ribose is substituted with methoxy and the phosphate-binding region is a phosphorothioate bond:



wherein Base represents a nucleobase.

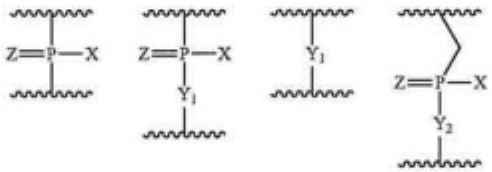
The oligonucleotide of the present invention may be easily synthesized using various automated synthesizer (e.g., AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis may also be entrusted to a third-party organization (e.g., Promega Inc., or Takara Co.), etc.

The morpholino oligomer of the present invention is the oligomer of the present invention comprising the constituent unit represented by general formula below:



wherein Base has the same significance as defined above, and,

W represents a group shown by any one of the following groups:



wherein

X represents —CH_2R^1, —O—CH_2R^1, —S—CH_2R^1, —NR_2R^3 or F;

R^1 represents H or an alkyl;

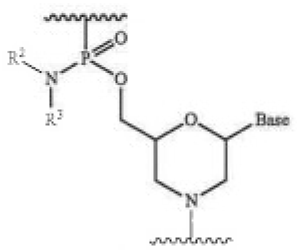
R^2 and R^3, which may be the same or different, each represents H, an alkyl, a cycloalkyl or an aryl;

Y_1 represents O, S, CH_2 or NR^1;

Y_2 represents O, S or NR^1;

Z represents O or S.

Preferably, the morpholino oligomer is an oligomer comprising a constituent unit represented by general formula below (phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).



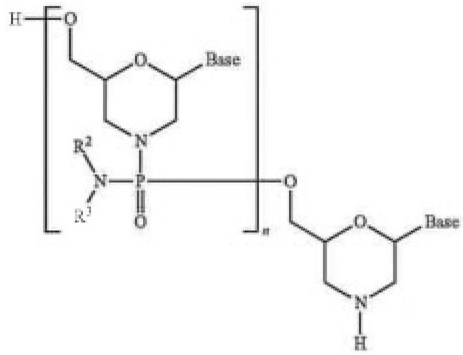
wherein Base, R^2 and R^3 have the same significance as defined above.

The morpholino oligomer may be produced in accordance with, e.g., WO 1991/009033 or WO 2009/064471. In particular, PMO can be produced by the procedure described in WO 2009/064471 or produced by the process shown below.

[Method for Producing PMO]

An embodiment of PMO is, for example, the compound represented by general formula (I) below (hereinafter PMO (I)).

(I)



wherein

Base, R^2 and R^3 have the same significance as defined above; and,

n is a given integer of 1 to 99, preferably a given integer of 18 to 28.

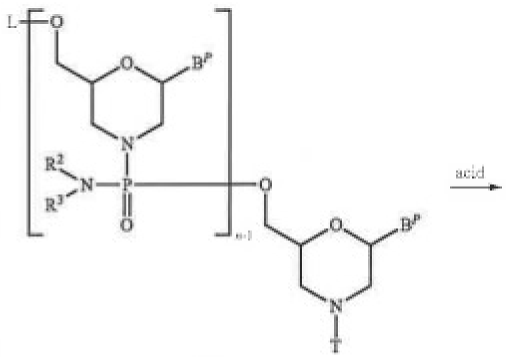
PMO (I) can be produced in accordance with a known method, for example, can be produced by performing the procedures in the following steps.

The compounds and reagents used in the steps below are not particularly limited so long as they are commonly used to prepare PMO.

Also, the following steps can all be carried out by the liquid phase method or the solid phase method (using manuals or commercially available solid phase automated synthesizers). In producing PMO by the solid phase method, it is desired to use automated synthesizers in view of simple operation procedures and accurate synthesis.

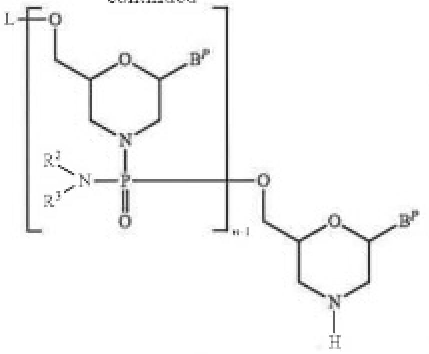
(1) Step A:

The compound represented by general formula (II) below (hereinafter referred to as Compound (II)) is reacted with an acid to prepare the compound represented by general formula (III) below (hereinafter referred to as Compound (III)):



(II)

-continued



(III)

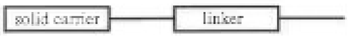
wherein n, R^2 and R^3 have the same significance as defined above;

each B^P independently represents a nucleobase which may optionally be protected;

T represents trityl, monomethoxytrityl or dimethoxytrityl; and,

L represents hydrogen, an acyl or a group represented by general formula (IV) below (hereinafter referred to as group (IV)).

(IV)



The "nucleobase" for B^P includes the same "nucleobase" as in Base, provided that the amino or hydroxy group in the nucleobase shown by B^P may be protected.

Such protective group for amino is not particularly limited so long as it is used as a protective group for nucleic acids. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-tert-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Specific examples of the protective group for the hydroxy group include 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, and phenyl, which may be substituted by 1 to 5 electron-withdrawing group at optional substitutable positions, diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrolidinylcarbamoyl, morpholinocarbamoyl, 4-(tert-butylcarboxy)benzyl, 4-[(dimethylamino)carboxy]benzyl and 4-(phenylcarboxy)benzyl, (cf., e.g., WO 2009/064471).

The "solid carrier" is not particularly limited so long as it is a carrier usable for the solid phase reaction of nucleic acids. It is desired for the solid carrier to have the following properties: e.g., (i) it is sparingly soluble in reagents that can be used for the synthesis of morpholino nucleic acid derivatives (e.g., dichloromethane, acetonitrile, tetrazole, N-methylimidazole, pyridine, acetic anhydride, lutidine, trifluoroacetic acid); (ii) it is chemically stable to the reagents usable for the synthesis of morpholino nucleic acid derivatives; (iii) it can be chemically modified; (iv) it can be charged with desired morpholino nucleic acid derivatives; (v) it has a strength sufficient to withstand high pressure through treatments; and (vi) it has a uniform particle diameter range and distribution. Specifically, swellable polystyrene (e.g., aminomethyl polystyrene resin 1% dibenzylbenzene crosslinked (200-400 mesh) (2.4-3.0 mmol/g) (manufactured by Tokyo Chemical Industry), Aminomethylated Polystyrene Resin.HCl [dibenzylbenzene 1%, 100-200 mesh] (manufactured by Peptide Institute, Inc.)), non-swellable polystyrene (e.g., Primer Support (manufactured by GE Healthcare)), PEG chain-attached polystyrene (e.g., NH_2—PEG resin (manufactured by Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured by, e.g., CPG), oxalyl-controlled pore glass (cf., e.g., Alul et al., Nucleic Acids Research, Vol. 19, 1527 (1991)), TentaGel support-aminopolyethylene glycol-derivatized support (e.g., Wright et al., cf., Tetrahedron Letters, Vol. 34, 3373 (1993)), and a copolymer of Poros-polystyrene/divinylbenzene.

A "linker" which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives. Examples include 3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA).

This step can be performed by reacting Compound (II) with an acid.

The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

An organic amine can be used in combination with the acid described above. The organic amine is not particularly limited and includes, for example, triethylamine. The amount of the organic amine used is appropriately in a range of, e.g., 0.01 mol equivalent to 10 mol equivalents, and preferably in a range of 0.1 mol equivalent to 2 mol equivalents, based on 1 mol of the acid.

When a salt or mixture of the acid and the organic amine is used in this step, the salt or mixture includes, for example, a salt or mixture of trifluoroacetic acid and triethylamine, and more specifically, a mixture of 1 equivalent of triethylamine and 2 equivalents of trifluoroacetic acid.

The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

The reaction temperature in the reaction described above is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the acid used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

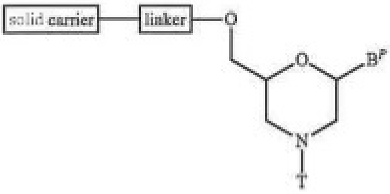
After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, diisopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

The solvent used in this step is not particularly limited so long as it is inert to the reaction, and includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and a mixture thereof. The reaction temperature is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

In Compound (II), the compound of general formula (IIa) below (hereinafter Compound (IIa)), wherein n is 1 and L is a group (IV), can be produced by the following procedure.

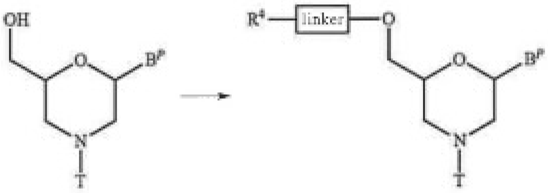
(IIa)



wherein B^P, T, linker and solid carrier have the same significance as defined above.

Step 1

The compound represented by general formula (V) below is reacted with an acylating agent to prepare the compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).



(V) (VI)

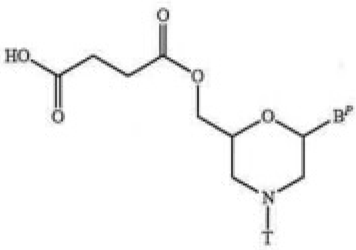
wherein B^P, T and linker have the same significance as defined above; and,

R^4 represents hydroxy, a halogen or amino.

This step can be carried out by known procedures for introducing linkers, using Compound (V) as the starting material.

In particular, the compound represented by general formula (VIa) below can be produced by performing the method known as esterification, using Compound (V) and succinic anhydride.

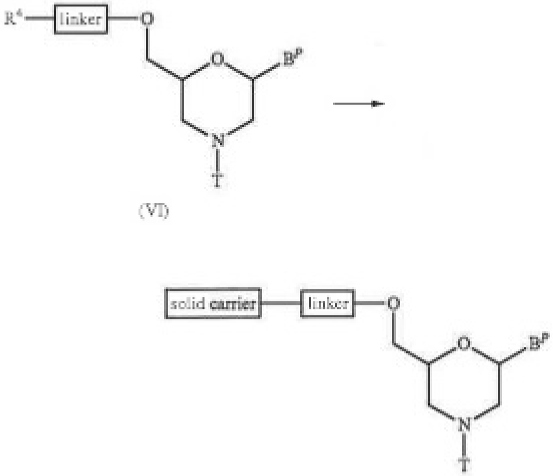
(VIa)



wherein B^P and T have the same significance as defined above.

Step 2

Compound (VI) is reacted with a solid career by a condensing agent to prepare Compound (IIa).

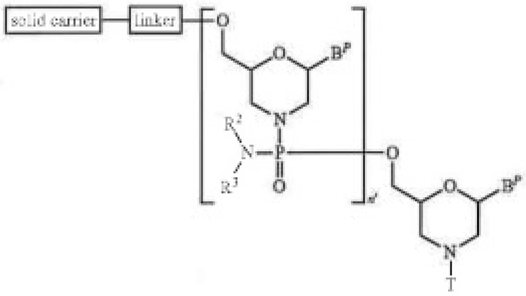


wherein B^P, R^4, T, linker and solid carrier have the same significance as defined above.

This step can be performed using Compound (VI) and a solid carrier in accordance with a process known as condensation reaction.

In Compound (II), the compound represented by general formula (IIa2) below wherein n is 2 to 99 and L is a group represented by general formula (IV) can be produced by using Compound (IIa) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

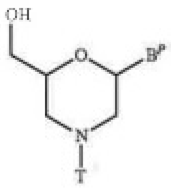
(IIa2)



wherein B^P, R^2, R^3, T, linker and solid carrier have the same significance as defined above; and,

n' represents 1 to 98.

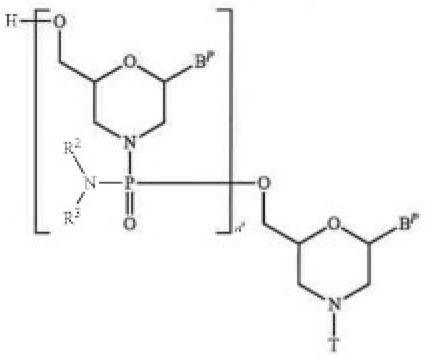
In Compound (II), the compound of general formula (IIb) below wherein n is 1 and L is hydrogen can be produced by the procedure described in, e.g., WO 1991/009033.



(IIb)

wherein B^P and T have the same significance as defined above.

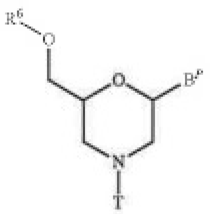
In Compound (II), the compound represented by general formula (IIb2) below wherein n is 2 to 99 and L is hydrogen can be produced by using Compound (IIb) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.



(IIb2)

wherein B^P, n', R^2, R^3 and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIc) below wherein n is 1 and L is an acyl can be produced by performing the procedure known as acylation reaction, using Compound (IIb).

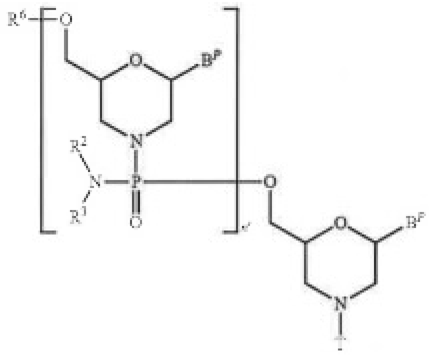


(IIc)

wherein B^P and T have the same significance as defined above; and,

R^5 represents an acyl.

In Compound (II), the compound represented by general formula (IIc2) below wherein n is 2 to 99 and L is an acyl can be produced by using Compound (IIc) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

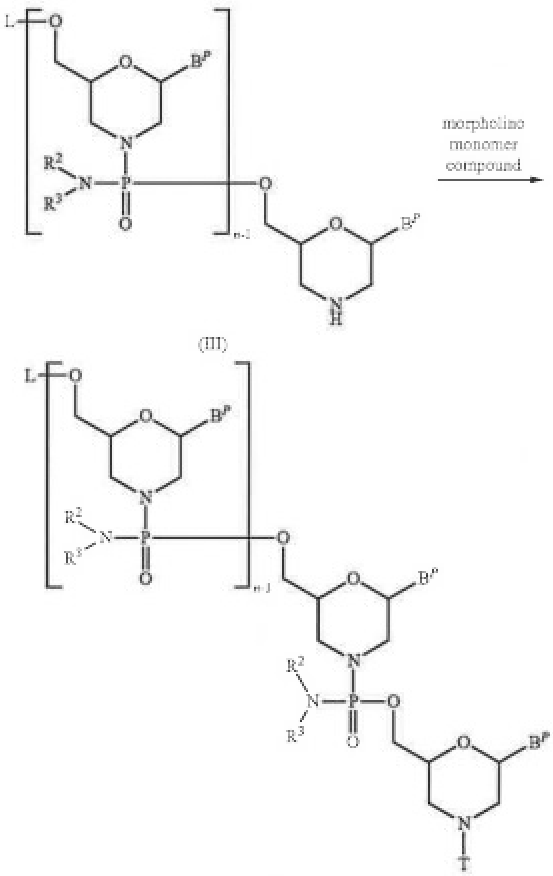


(IIc2)

wherein B^P, n', R^2, R^3, R^5 and T have the same significance as defined above.

(2) Step B

Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):



(III)

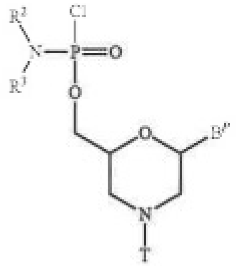
(VII)

wherein B^P, L, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by reacting Compound (III) with the morpholino monomer compound in the presence of a base.

The morpholino monomer compound includes, for example, compounds represented by general formula (VIII) below:

(VIII)



wherein B^P, R^2, R^3 and T have the same significance as defined above.

The "base" which can be used in this step includes, for example, diisopropylamine, triethylamine and N-ethylmorpholine. The amount of the base used is appropriately in a range of 1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (III), preferably, 10 mol equivalents to 100 mol equivalents based on 1 mol of Compound (III).

The morpholino monomer compound and base which can be used in this step may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, N,N-dimethylimidazolidone, N-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, or a mixture thereof.

The reaction temperature is preferably in a range of, e.g., 0° C. to 100° C., and more preferably, in a range of 10° C. to 50° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 1 minute to 48 hours in general, and preferably in a range of 30 minutes to 24 hours.

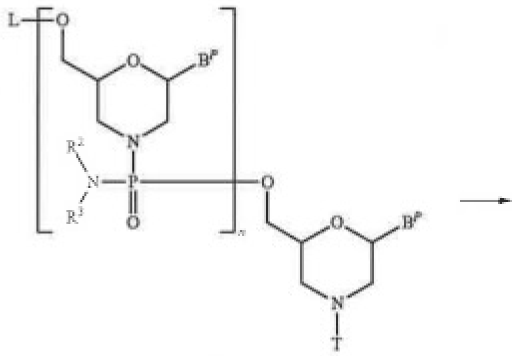
Furthermore, after completion of this step, an acylating agent can be added, if necessary. The "acylating agent" includes, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol(s) (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine, N-ethylmorpholine, etc. may also be used in combination with the acylating agent. The amount of the acylating agent is appropriately in a range of 0.1 mol equivalent to 10000 mol equivalents, and preferably in a range of 1 mol equivalent to 1000 mol equivalents. The amount of the base is appropriately in a range of, e.g., 0.1 mol equivalent to 100 mol equivalents, and preferably in a range of 1 mol equivalent to 10 mol equivalents, based on 1 mol of the acylating agent.

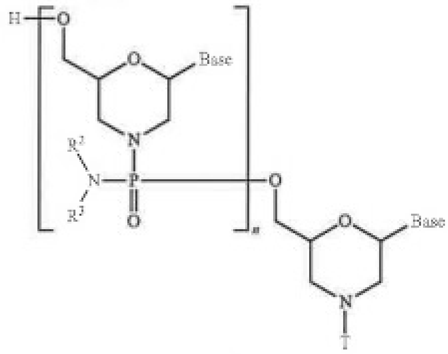
The reaction temperature in this reaction is preferably in a range of 10° C. to 50° C., more preferably, in a range of 10° C. to 50° C., much more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C. The reaction time may vary depending upon kind of the acylating agent used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

(3) Step C:

In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



(VII)



(IX)

wherein Base, B^P, n, R^2, R^3 and T have the same significance as defined above.

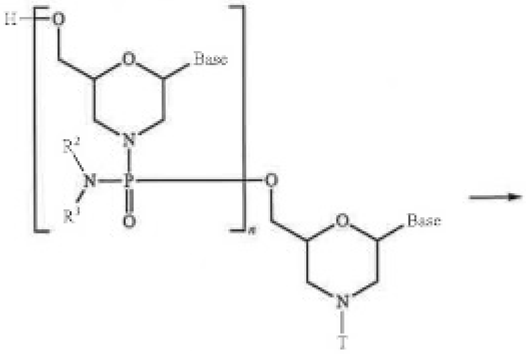
This step can be performed by reacting Compound (VII) with a deprotecting agent.

The "deprotecting agent" includes, e.g., conc. ammonia water and methylamine. The "deprotecting agent" used in this step may also be used as a dilution with, e.g., water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-dimethylimidazolidone, N-methylpiperidone, or a mixture of these solvents. Among others, ethanol is preferred. The amount of the deprotecting agent used is appropriately in a range of, e.g., 1 mol equivalent to 100000 mol equivalents, and preferably in a range of 10 mol equivalents to 1000 mol equivalents, based on 1 mol of Compound (VII).

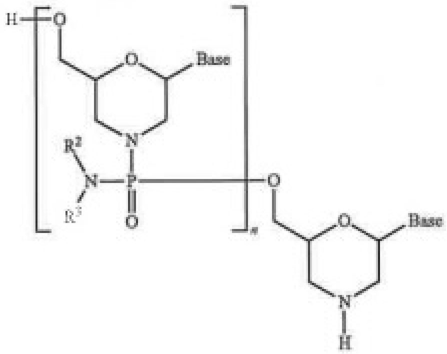
The reaction temperature is appropriately in a range of 15° C. to 75° C., preferably, in a range of 40° C. to 70° C., and more preferably, in a range of 50° C. to 60° C. The reaction time for deprotection may vary depending upon kind of Compound (VII), reaction temperature, etc., and is appropriately in a range of 10 minutes to 30 hours, preferably 30 minutes to 24 hours, and more preferably in a range of 5 hours to 20 hours.

(4) Step D:

PMO (I) is produced by reacting Compound (IX) produced in step C with an acid:



(IX)



(I)

wherein Base, n, R^2, R^3 and T have the same significance as defined above. This step can be performed by adding an acid to Compound (IX).

The "acid" which can be used in this step includes, for example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid, hydrochloric acid, etc. The acid used is appropriately used to allow the solution to have a pH range of 0.1 to 4.0, and more preferably, in a range of pH 1.0 to 3.0. The solvent is not particularly limited so long as it is inert to the reaction, and includes, for example, acetonitrile, water, or a mixture of these solvents thereof.

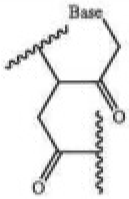
The reaction temperature is appropriately in a range of 10° C. to 50° C., preferably, in a range of 20° C. to 40° C., and more preferably, in a range of 25° C. to 35° C. The reaction time for deprotection may vary depending upon kind of Compound (IX), reaction temperature, etc., and is appropriately in a range of 0.1 minute to 5 hours, preferably 1 minute to 1 hour, and more preferably in a range of 1 minute to 30 minutes.

PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C_8 to C_{18}, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., e.g., WO 1991/09033).

In purification of PMO (I) using reversed phase chromatography, e.g., a solution mixture of 20 mM triethylamine/acetate buffer and acetonitrile can be used as an elution solvent.

In purification of PMO (I) using ion exchange chromatography, e.g., a solution mixture of 1 M saline solution and 10 mM sodium hydroxide aqueous solution can be used as an elution solvent.

A peptide nucleic acid is the oligomer of the present invention having a group represented by the following general formula as the constituent unit:



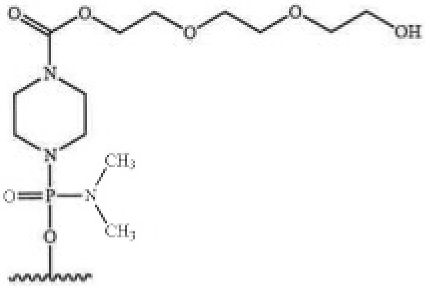
wherein Base has the same significance as defined above.

Peptide nucleic acids can be prepared by referring to, e.g., the following literatures.

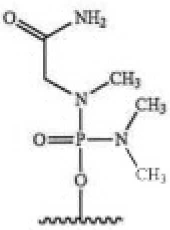
1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)
2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)
3) K. L. Dueholmn, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)
4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J. Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)
5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G. Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

In the oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH.

(1)



(2)



(3)

Hereinafter, the groups shown by (1), (2) and (3) above are referred to as "Group (1)," "Group (2)" and "Group (3)," respectively.

2. Pharmaceutical Composition

The oligomer of the present invention causes exon 53 skipping with a higher efficiency as compared to the prior art

antisense oligomers. It is thus expected that conditions of muscular dystrophy can be relieved with high efficience by administering the pharmaceutical composition comprising the oligomer of the present invention to DMD patients. For example, when the pharmaceutical composition comprising the oligomer of the present invention is used, the same therapeutic effects can be achieved even in a smaller dose than that of the oligomers of the prior art. Accordingly, side effects can be alleviated and such is economical.

In another embodiment, the present invention provides the pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the oligomer of the present invention, a pharmaceutically acceptable salt or hydrate thereof (hereinafter referred to as "the composition of the present invention").

Examples of the pharmaceutically acceptable salt of the oligomer of the present invention contained in the composition of the present invention are alkali metal salts such as salts of sodium, potassium and lithium; alkaline earth metal salts such as salts of calcium and magnesium; metal salts such as salts of aluminum, iron, zinc, copper, nickel, cobalt, etc.; ammonium salts; organic amine salts such as salts of t-octylamine, dibenzylamine, morpholine, glucosamine, phenylglycine alkyl ester, ethylenediamine, N-methylglucamine, guanidine, diethylamine, triethylamine, dicyclohexylamine, N,N-dibenzylethylenediamine, chloroprocaine, procaine, diethanolamine, N-benzylphenethylamine, piperazine, tetramethylammonium, tris(hydroxymethyl)aminomethane; hydrohalide salts such as salts of hydrofluorates, hydrochlorides, hydrobromides and hydroiodides; inorganic acid salts such as nitrates, perchlorates, sulfates, phosphates, etc.; lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates; arylsulfonates such as benzenesulfonates and p-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartarates, oxalates, maleates, etc.; and, amino acid salts such as salts of glycine, lysine, arginine, ornithine, glutamic acid and aspartic acid. These salts may be produced by known methods. Alternatively, the oligomer of the present invention contained in the composition of the present invention may be in the form of a hydrate thereof.

Administration route for the composition of the present invention is not particularly limited so long as it is pharmaceutically acceptable route for administration, and can be chosen depending upon method of treatment. In view of easiness in delivery to muscle tissues, preferred are intravenous administration, intraarterial administration, intramuscular administration, subcutaneous administration, oral administration, tissue administration, transdermal administration, etc. Also, dosage forms which are available for the composition of the present invention are not particularly limited, and include, for example, various injections, oral agents, drips, inhalations, ointments, lotions, etc.

In administration of the oligomer of the present invention to patients with muscular dystrophy, the composition of the present invention preferably contains a carrier to promote delivery of the oligomer to muscle tissues. Such a carrier is not particularly limited as far as it is pharmaceutically acceptable, and examples include cationic carriers such as cationic liposomes, cationic polymers, etc., or carriers using viral envelope. The cationic liposomes are, for example, liposomes composed of 2-O-(2-diethylaminoethyl)carabamoyl-1,3-O-dioleoylglycerol and phospholipids as the essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (manufactured by Invitrogen Corp.), DMRIE-C (registered trademark) (manufactured by Invitrogen Corp.), GeneSilencer (registered trademark) (manufactured by Gene Therapy Systems), TransMessenger (registered trademark) (manufactured by QIAGEN, Inc.), TransIT TKO (registered trademark) (manufactured by Mirus) and Nucleofector II (Lonza). Among others, liposome A is preferred. Examples of cationic polymers are JetSI (registered trademark) (manufactured by Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethylenimine, manufactured by Qbiogene, Inc.). An example of carriers using viral envelop is GenomeOne (registered trademark) (HVJ-E liposome, manufactured by Ishihara Sangyo). Alternatively, the medical devices described in Japanese Patent No. 2924179 and the cationic carriers described in Japanese Domestic Re-Publication PCT Nos. 2006/129594 and 2008/096690 may be used as well.

A concentration of the oligomer of the present invention contained in the composition of the present invention may vary depending on kind of the carrier, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the oligomer of the present invention contained in the composition of the present invention and the carrier (carrier/oligomer of the present invention) may vary depending on property of the oligomer, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20.

In addition to the oligomer of the present invention and the carrier described above, pharmaceutically acceptable additives may also be optionally formulated in the composition of the present invention. Examples of such additives are emulsification aids (e.g., fatty acids having 6 to 22 carbon atoms and their pharmaceutically acceptable salts, albumin and dextran), stabilizers (e.g., cholesterol and phosphatidic acid), isotonizing agents (e.g., sodium chloride, glucose, maltose, lactose, sucrose, trehalose), and pH controlling agents (e.g., hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine). One or more of these additives can be used. The content of the additive in the composition of the present invention is appropriately 90 wt % or less, preferably 70 wt % or less and more preferably, 50 wt % or less.

The composition of the present invention can be prepared by adding the oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may be added at an appropriate step either before or after addition of the oligomer of the present invention. An aqueous solvent that can be used in adding the oligomer of the present invention is not particularly limited as far as it is pharmaceutically acceptable, and examples are injectable water or injectable distilled water, electrolyte fluid such as physiological saline, etc., and sugar fluid such as glucose fluid, maltose fluid, etc. A person skilled in the art can appropriately choose conditions for pH and temperature for such matter.

The composition of the present invention may be prepared into, e.g., a liquid form and its lyophilized preparation. The lyophilized preparation can be prepared by lyophilizing the composition of the present invention in a liquid form in a conventional manner. The lyophilization can be performed, for example, by appropriately sterilizing the composition of the present invention in a liquid form, dispensing an aliquot into a vial container, performing preliminary freezing for 2 hours at conditions of about −40 to −20° C., performing a primary drying at 0 to 10° C. under reduced pressure, and then performing a secondary drying at about 15 to 25° C. under reduced pressure. In general, the lyophilized prepa-

ration of the composition of the present invention can be obtained by replacing the content of the vial with nitrogen gas and capping.

The lyophilized preparation of the composition of the present invention can be used in general upon reconstitution by adding an optional suitable solution (reconstitution liquid) and redissolving the preparation. Such a reconstitution liquid includes injectable water, physiological saline and other infusion fluids. A volume of the reconstitution liquid may vary depending on the intended use, etc., is not particularly limited, and is suitably 0.5 to 2-fold greater than the volume prior to lyophilization or no more than 500 mL.

It is desired to control a dose of the composition of the present invention to be administered, by taking the following factors into account: the type and dosage form of the oligomer of the present invention contained; patients' conditions including age, body weight, etc.; administration route; and the characteristics and extent of the disease. A daily dose calculated as the amount of the oligomer of the present invention is generally in a range of 0.1 mg to 10 g/human, and preferably 1 mg to 1 g/human. This numerical range may vary occasionally depending on type of the target disease, administration route and target molecule. Therefore, a dose lower than the range may be sufficient in some occasion and conversely, a dose higher than the range may be required occasionally. The composition can be administered from once to several times daily or at intervals from one day to several days.

In still another embodiment of the composition of the present invention, there is provided a pharmaceutical composition comprising a vector capable of expressing the oligonucleotide of the present invention and the carrier described above. Such an expression vector may be a vector capable of expressing a plurality of the oligonucleotides of the present invention. The composition may be formulated with pharmaceutically acceptable additives as in the case with the composition of the present invention containing the oligomer of the present invention. A concentration of the expression vector contained in the composition may vary depending upon type of the career, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the expression vector contained in the composition and the carrier (carrier/expression vector) may vary depending on property of the expression vector, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20. The content of the carrier contained in the composition is the same as in the case with the composition of the present invention containing the oligomer of the present invention, and a method for producing the same is also the same as in the case with the composition of the present invention.

Hereinafter, the present invention will be described in more detail with reference to EXAMPLES and TEST EXAMPLES below, but is not deemed to be limited thereto.

EXAMPLES

[Reference Example 1] 4-{[(2S,6R)-(4-amido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl] methoxy}-4-oxobutanoic acid laded onto aminomethyl polystyrene resin

Step 1: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl] meth oxy}-4-oxobutanoic acid

Under argon atmosphere, 22.0 g of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichloromethane, and 5.76 g of succinic anhydride was added to the suspension, followed by stirring at room temperature for 3 hours. To the reaction solution was added 40 mL of methanol, and the mixture was concentrated under reduced pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogenphosphate solution. The resulting organic layer was washed sequentially with 0.5M aqueous potassium dihydrogenphosphate solution, water and brine in the order mentioned. The resulting organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 25.9 g of the product.

Step 2: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl] methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

After 23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the solution. Then, 25.0 g of Aminomethyl Polystyrene Resin cross-linked with 1% DVB (manufactured by Tokyo Chemical Industry Co., Ltd., A1543) and 24 mL of triethylamine were added to the mixture, followed by shaking at room temperature for 4 days. After completion of the reaction, the resin was taken out by filtration. The resulting resin was washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure. To the resulting resin were added 150 mL of tetrahydrofuran (dehydrate), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine, and the mixture was shaken at room temperature for 2 hours. The resin was taken out by filtration, washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure to give 33.7 g of the product.

The loading amount of the product was determined by measuring UV absorbance at 409 nm of the molar amount of the trityl per g resin using a known method. The loading amount of the resin was 397.4 μmol/g.

Conditions of UV Measurement

Device: U-2910 (Hitachi, Ltd.)

Solvent: methanesulfonic acid

Wavelength: 265 nm

ε Value: 45000

[Reference Example 2] 4-Oxo-4-{[2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto 2-aminomethylpolystyrene resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

Guanosine, 100 g, was dried at 80° C. under reduced pressure for 24 hours. After 500 mL of pyridine (anhydrous) and 500 mL of dichloromethane (anhydrous) were added thereto, 401 mL of chlorotrimethylsilane was dropwise added to the mixture under an argon atmosphere at 0° C., followed by stirring at room temperature for 3 hours. The mixture was again ice-cooled and 66.3 g of phenoxyacetyl chloride was dropwise added thereto. Under ice cooling, the mixture was stirred for further 3 hours. To the reaction solution was added 500 mL of methanol, and the mixture

was stirred at room temperature overnight. The solvent was then removed by distillation under reduced pressure. To the residue was added 500 mL of methanol, and concentration under reduced pressure was performed 3 times. To the residue was added 4 L of water, and the mixture was stirred for an hour under ice cooling. The precipitates formed were taken out by filtration, washed sequentially with water and cold methanol and then dried to give 150.2 g of the objective compound (yield: 102%) (cf.: Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2: N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide p-toluenesulfonate

In 480 mL of methanol was suspended 30 g of the compound obtained in Step 1, and 130 mL of 2N hydrochloric acid was added to the suspension under ice cooling. Subsequently, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added to the mixture in the order mentioned and stirred at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matters were removed by filtration, followed by washing with 100 mL of methanol. The filtrate and washing liquid were combined and the mixture was ice cooled. To the mixture was added 11.52 g of 2-picoline borane. After stirring for 20 minutes, 54.6 g of p-toluenesulfonic acid monohydrate was slowly added to the mixture, followed by stirring at 4° C. overnight. The precipitates were taken out by filtration and washed with 500 mL of cold methanol and dried to give 17.7 g of the objective compound (yield: 43.3%).

^{1}H NMR (δ, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J=7.82 Hz), 7.00 (3H, m), 5.95 (1H, dd, J=10.64, 2.42 Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H, m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

In 30 mL of dichloromethane was suspended 2.0 g of the compound obtained in Step 2, and 13.9 g of triethylamine and 18.3 g of trityl chloride were added to the suspension under ice cooling. The mixture was stirred at room temperature for an hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried. The organic layer was concentrated under reduced pressure. To the residue was added 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)), and the mixture was stirred. Subsequently, 40 mL of water was added and the mixture was stirred for an hour under ice cooling. The mixture was taken out by filtration, washed with cold methanol and dried to give 1.84 g of the objective compound (yield: 82.0%).

Step 4: Production of 4-oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[Reference Example 3] 4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4(1H,3H)-dione was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[Reference Example 4] 1,12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 2-[2-(2-hydroxyethoxyl)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471) was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide.

According to the descriptions in EXAMPLES 1 to 12 and REFERENCE EXAMPLES 1 to 3 below, various types of PMO shown by PMO Nos. 1-11 and 13-16 in TABLE 2 were synthesized. The PMO synthesized was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

TABLE 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31-55	5' end: group (3)	SEQ ID NO: 4
2	32-53	5' end: group (3)	SEQ ID NO: 8
3	32-56	5' end: group (3)	SEQ ID NO: 11
4	33-54	5' end: group (3)	SEQ ID NO: 15
5	34-58	5' end: group (3)	SEQ ID NO: 25
6	36-53	5' end: group (3)	SEQ ID NO: 32
7	36-55	5' end: group (3)	SEQ ID NO: 34
8	36-56	5' end: group (3)	SEQ ID NO: 35
9	36-57	5' end: group (3)	SEQ ID NO: 36

TABLE 2-continued

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
10	33-57	5' end: group (3)	SEQ ID NO: 18
11	39-69	Sequence corresponding to H53A(+39 + 69) (cf. Table 1) in Non-Patent Document 3, 5' end: group (3)	SEQ ID NO: 38
12	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5, 5' end: group (2)	SEQ ID NO: 39
13	32-56	5' end: group (1)	SEQ ID NO: 11
14	36-56	5' end: group (1)	SEQ ID NO: 35
15	30-59	Sequence corresponding to h53A30/1 cf. Table 1) in Non-Patent Document 5 5' end: group (3)	SEQ ID NO: 39
16	23-47	Sequence corresponding to SEQ ID NO: 429 described in Patent Document 4, 5' end: group (3)	SEQ ID NO: 47

Example 1

PMO No. 8

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmol) was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. The mixture was allowed to stand for 30 minutes. After the mixture was further washed twice with 30 mL of dichloromethane, the following synthesis cycle was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (min)
1	deblocking solution	30	2.0
2	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6-11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromethane	30	0.5
25	dichloromethane	30	0.5

The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained by dissolving the morpholino monomer compound in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15M. The coupling solution B used was a solution obtained by dissolving N,N-diisopropylethylamine in 1,3-dimethyl-2-imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2,6-lutidine in dichloromethane.

The aminomethyl polystyrene resin loaded with the PMO synthesized above was recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55° C. for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. The resulting residue was dissolved in 100 mL of a solvent mixture of 20 mM acetic acid-triethylamine buffer (TEAA buffer) and acetonitrile (4/1) and filtered through a membrane filter. The filtrate obtained was purified by reversed phase HPLC. The conditions used are as follows.

TABLE 4

Column	XTerra MS18 (Waters, φ50 × 100 mm, 1CV = 200 mL)
Flow rate	60 mL/min
Column temperature	room temperature
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20 → 50%/9CV

Each fraction was analyzed and the product was recovered in 100 mL of acetonitrile-water (1/1), to which 200 mL of ethanol was added. The mixture was concentrated under reduced pressure. Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL of 10 mM phosphoric acid aqueous solution to suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45 μm). The mixture was thoroughly washed

with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution.

The resulting aqueous solution containing the product was purified by an anionic exchange resin column. The conditions used are as follows.

TABLE 5

Column	Source 30Q (GE Healthcare, φ 40 × 150 mm, 1CV = 200 mL)
Flow rate	80 mL/min
Column temp.	room temperature
Solution A	10 mM sodium hydroxide aqueous solution
Solution B	10 mM sodium hydroxide aqueous solution, 1M sodium chloride aqueous solution
Gradient	(B) conc. 5 → 35%/15CV

Each fraction was analyzed (on HPLC) and the product was obtained as an aqueous solution. To the resulting aqueous solution was added 225 mL of 0.1M phosphate buffer (pH 6.0) for neutralization. The mixture was filtered through a membrane filter (0.45 μm). Next, ultrafiltration was performed under the conditions

TABLE 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

The filtrate was concentrated to give approximately 250 mL of an aqueous solution. The resulting aqueous solution was filtered through a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried to give 1.5 g of the objective compound as a white cotton-like solid.

ESI-TOF-MS Calcd.: 6924.82.

Found: 6923.54.

Example 2

PMO. No. 1

The title compound was produced in accordance with the procedure of EXAMPLE 1.

MALDI-TOF-MS Calcd.: 8291.96.

Found: 8296.24.

Example 3

PMO. No. 2

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 7310.13.

Found: 7309.23.

Example 4

PMO. No. 3

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8270.94.

Found: 8270.55.

Example 5

PMO. No. 4

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-(2H)-yl)-4-tritylmorpholin-2-yl)methoxy)-4-oxobutanoic acid (REFERENCE EXAMPLE 3) loaded onto aminomethyl polystyrene resin was used as the starting material.

ESI-TOF-MS Calcd.: 7310.13.

Found: 7310.17.

Example 6

PMO. No. 5

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl)methoxy)-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 3) was used as the starting material.

ESI-TOF-MS Calcd.: 8270.94.

Found: 8270.20.

Example 7

PMO. No. 6

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 5964.01.

Found: 5963.68.

Example 8

PMO. No. 7

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 6609.55.

Found: 6608.85.

Example 9

PMO. No. 9

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 7280.11.

Found: 7279.4.

Example 10

PMO. No. 10

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-

6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 8295.95.

Found: 8295.91.

Example 11

PMO. No. 13

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 7276.15.

Found: 7276.69.

Example 12

PMO. No. 14

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-5-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 8622.27.

Found: 8622.29.

Comparative Example 1

PMO. No. 11

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 10274.63.

Found: 10273.71.

Comparative Example 2

PMO. No. 15

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 9941.33.

Found: 9940.77.

Comparative Example 3

PMO. No. 16

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8238.94.

Found: 8238.69.

Test Example 1

In Vitro Assay

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 10 μM of the oligomers PMO Nos. 1 to 8 of the present invention and the antisense oligomer PMO No. 11 were transfected with 4×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

```
Forward primer:
                                    (SEQ ID NO: 40)
5'-ACGATTTCGAACAGAGGCGTC-3'

Reverse primer:
                                    (SEQ ID NO: 41)
5'-GTCTGCCACTGGCGGAGGTC-3'
```

Next, a nested PCR was performed with the product amplified by RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

```
Forward primer:
                                    (SEQ ID NO: 42)
5'-CATCAAGCAGAAGGCAACAA-3'

Reverse primer:
                                    (SEQ ID NO: 43)
5'-GAAGTTTCAGGGCCAAGTCA-3'
```

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)}=A/(A+B)\times100$$

Experimental Results

The results are shown in FIG. 1. This experiment revealed that the oligomers PMO Nos. 1 to 8 of the present invention

caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomer PMO No. 11. In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than four times higher exon skipping efficiency than that of the antisense oligomer PMO No. 11.

Test Example 2

In Vitro Assay Using Human Fibroblasts

Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (human normal tissue-derived fibroblasts, National Institute of Biomedical Innovation) or 5017 cells (human DMD patient-derived fibroblasts, Coriell Institute for Medical Research) using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at 5×10^4/cm^2 into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM.F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12 to 14 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and the morpholino oligomer was added thereto in a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEX51F and hEX55R.

```
hEX51F:
                              (SEQ ID NO: 45)
5'-CGGGCTTGGACAGAACTTAC-3'

hEx55R:
                              (SEQ ID NO: 46)
5'-TCCTTACGGGTAGCATCCTG-3'
```

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency }(\%)=A/(A+B)\times100$$

Experimental Results

The results are shown in FIGS. **2** and **3**. This experiment revealed that in TIG-119 cells, the oligomers PMO Nos. 3, 8 and 9 of the present invention (FIG. **2**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **2**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than twice higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **2**).

Furthermore, this experiment revealed that the oligomers PMO Nos. 3 and 8 to 10 of the present invention (FIG. **3**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **3**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than seven times higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **3**).

Test Example 3

In Vitro Assay Using Human Fibroblasts

The skin fibroblast cell line (fibroblasts from human DMD patient (exons 45-52 or exons 48-52)) was established by biopsy from the medial left upper arm of DMD patient with deletion of exons 45-52 or DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at 5×10^4/cm^2 into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12, 14 or 20 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto at a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEx44F and h55R.

```
hEx44F:
                              (SEQ ID NO: 48)
5'-TGTTGAGAAATGGCGGCGT-3'

hEx55R:
                              (SEQ ID NO: 46)
5'-TCCTTACGGGTAGCATCCTG-3'
```

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)}=A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. 4 and 5. This experiment revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with an efficiency as high as more than 80% in the cells from DMD patient with deletion of exons 45-52 (FIG. 4) or deletion of exons 48-52 (FIG. 5). Also, the oligomers PMO Nos. 3 and 8 of the present invention were found to cause exon 53 skipping with a higher efficiency than that of the antisense oligomer PMO No. 15 in the cells from DMD patient with deletion of exons 45-52 (FIG. 4).

Test Example 4

Western Blotting

The oligomer PMO No. 8 of the present invention was added to the cells at a concentration of 10 μM, and proteins were extracted from the cells after 72 hours using a RIPA buffer (manufactured by Thermo Fisher Scientific) containing Complete Mini (manufactured by Roche Applied Science) and quantified using a BCA protein assay kit (manufactured by Thermo Fisher Scientific). The proteins were electrophoresed in NuPAGE Novex Tris-Acetate Gel 3-8% (manufactured by Invitrogen) at 150V for 75 minutes and transferred onto a PVDF membrane (manufactured by Millipore) using a semi-dry blotter. The PVDF membrane was blocked with a 5% ECL Blocking agent (manufactured by GE Healthcare) and the membrane was then incubated in a solution of anti-dystrophin antibody (manufactured by NCL-Dys1, Novocastra). After further incubation in a solution of peroxidase-conjugated goat-antimouse IgG (Model No. 170-6516, Bio-Rad), the membrane was stained with ECL Plus Western blotting system (manufactured by GE Healthcare).

Immunostaining

The oligomer PMO No. 3 or 8 of the present invention was added to the cells. The cells after 72 hours were fixed in 3% paraformaldehyde for 10 minutes, followed by incubation in 10% Triton-X for 10 minutes. After blocking in 10% goat serum-containing PBS, the membrane was incubated in a solution of anti-dystrophin antibody (NCL-Dys1, Novocastra). The membrane was further incubated in a solution of anti-mouse IgG antibody (manufactured by Invitrogen). The membrane was mounted with Pro Long Gold Antifade reagent (manufactured by Invitrogen) and observed with a fluorescence microscope.

Experimental Results

The results are shown in FIGS. 6 and 7. In this experiment it was confirmed by western blotting (FIG. 6) and immunostaining (FIG. 7) that the oligomers PMO Nos. 3 and 8 of the present invention induced expression of the dystrophin protein.

Test Example 5

In Vitro Assay Using Human Fibroblasts

The experiment was performed as in TEST EXAMPLE 3.

Experimental Results

The results are shown in FIG. 8. This experiment revealed that in the cells from DMD patients with deletion of exons 45-52, the oligomers PMO Nos. 3 to 8 of the present invention caused exon 53 skipping with a higher efficiency than the oligomers PMO Nos. 13 and 14 of the present invention (FIG. 8).

Test Example 6

In Vitro Assay

Experiments were performed using the antisense oligomers of 2'-O-methoxy-phosphorothioates (2'-OMe-S-RNA) shown by SEQ ID NO: 49 to SEQ ID NO: 123. Various antisense oligomers used for the assay were purchased from Japan Bio Services. The sequences of various antisense oligomers are given below.

TABLE 7

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68

TABLE 7-continued

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
H53_101-125	GACCUGCUCAGCUUCUUCCUUAGCU	70
H53_106-130	CCUAAGACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73
H53_121-145	UUGGCUCUGGCCUGUCCUAAGACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUGACUCAAGCUUGGCUCUGG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78
H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGGACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAGGGACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGGACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGGAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	GAUUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92
H53_144-168	GACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUAGGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGGACCCUCCUUCCAUCGA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101
H53_33-57	GCCUCCGGUUCUGAAGGUGUUCUUG	102
H53_34-58	UGCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUGCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUGCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUGUUGCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUGCCUCCGGUUCUGAAGGUG	107
H53_40-64	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
H53_32-51	GGUUCUGAAGGUGUUCUUGU	110
H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_40-59	UUGCCUCCGGUUCUGAAGGU	113
H53_42-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUGA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53_45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

RD cells (human rhabdomyosarcomna cell line) were plated at 3×10^5 in a 6-well plate and cultured in 2 nil, of Eagle's minimal **essential medium (EMEM) (manufactured** by Sigma, Inc., **hereinafter the same)** contai**ning 10% fetal** calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2 overnight. Complexes of various antisense oligomers (Japan Bio Services) (1 μM) for exon 53 skipping and Lipofectamine 2000 (manufactured by Invitrogen Corp.) were prepared and 200 μl was added to RD cells where 1.8 mL of the medium was exchanged, to reach the final concentration of 100 nM.

After completion of the addition, the cells were cultured overnight. The cells were washed twice with PBS (manufactured by Nissui, hereafter the same) and then 500 μl of ISOGEN (manufactured by Nippon Gene) were added to the cells. After the cells were allowed to stand at room temperature for a few minutes for cell lysis, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed **with 400 ng** of the **extracted** total RNA using a Titan One **Tube** RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal **cycler. The** RT-PCR program used is as follows.

50° C.**, 30 mins:** reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

```
Forward primer:
                                    (SEQ ID NO: 42)
5'CATCAAGCAGAAGGCAACAA-3'

Reverse primer:
                                    (SEQ ID NO: 43)
5'-GAAGTTTCAGGGCCAAGTCA-3'
```

Subsequently, a nested PCR was performed with the amplified product of RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

```
Forward primer:
                                    (SEQ. ID NO: 40)
5'-AGGATTTGGAACAGAGGCGTC-3'

Reverse primer:
                                    (SEQ ID NO: 41)
5'-GTCTGCCACTGGCGGAGGTC-3'
```

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency } (\%) = A/(A+B) \times 100$$

Experimental Results

The results are shown in FIGS. **9** to **17**. These experiments revealed that, when the antisense oligomers were designed at exons 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping could be caused with a high efficiency.

Test Example 7

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 0.3 to 30 μM of the antisense oligomers were transfected with 3.5×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After the transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was then added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (manufactured by Qiagen, Inc.). A reaction solution was prepared in accordance with the protocol attached to the kit.

The thermal cycler used was a PTC-100 (manufactured by MJ Research). The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 30 seconds; 60° C., 30 seconds; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 10 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

```
Forward primer:
                                    (SEQ. ID NO: 42)
5'-CATCAAGCAGAAGGCAACAA-3'

Reverse primer:
                                    (SEQ. ID NO: 43)
5'-GAAGTTTCAGGGCCAAGTCA-3'
```

The reaction product, 1 μl, of the PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency } (\%) = A/(A+B) \times 100$$

Experimental Results

The results are shown in FIGS. **18** and **19**. These experiments revealed that the oligomer PMO No. 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomers PMO Nos. 15 and 16 (FIG. **18**). It was also revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **19**). These results showed that the sequences with —OH group at the 5' end provide a higher skipping efficiency even in the same sequences.

INDUSTRIAL APPLICABILITY

Experimental results in TEST EXAMPLES demonstrate that the oligomers of the present invention (PMO Nos. 1 to 10) all caused exon 53 skipping with a markedly high efficiency under all cell environments, as compared to the oligomers (PMO Nos. 11, 12, 15 and 16) in accordance with the prior art. The 5017 cells used in TEST EXAMPLE 2 are the cells isolated from DMD patients, and the fibroblasts used in TEST EXAMPLES 3 and 5 are exon 53 skipping target cells from DMD patients. Particularly in TEST EXAMPLES 3 and 5, the oligomers of the present invention show the exon 53 skipping efficiency of 90% or higher in the cells from DMD patients that are the target for exon 53 skipping. Consequently, the oligomers of the present invention can induce exon 53 skipping with a high efficiency, when DMD patients are administered.

Therefore, the oligomers of the present invention are extremely useful for the treatment of DMD.

Sequence Listing Free Text

SEQ ID NO: 2: synthetic nucleic acid

SEQ ID NO: 3: synthetic nucleic acid

SEQ ID NO: 4: synthetic nucleic acid

SEQ ID NO: 5: synthetic nucleic acid

SEQ ID NO: 6: synthetic nucleic acid

SEQ ID NO: 7: synthetic nucleic acid

SEQ ID NO: 8: synthetic nucleic acid

SEQ ID NO: 9: synthetic nucleic acid
SEQ ID NO: 10: synthetic nucleic acid
SEQ ID NO: 11: synthetic nucleic acid
SEQ ID NO: 12: synthetic nucleic acid
SEQ ID NO: 13: synthetic nucleic acid
SEQ ID NO: 14: synthetic nucleic acid
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SEQUENCE LISTING

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<400> SEQUENCE: 15

tccggttctg aaggtgttct tg 22

<210> SEQ ID NO 16
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 16

ctccggttct gaaggtgttc ttg 23

<210> SEQ ID NO 17
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 17

cctccggttc tgaaggtgtt cttg 24

<210> SEQ ID NO 18
<211> LENGTH: 25
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 18

gcctccggtt ctgaaggtgt tcttg 25

<210> SEQ ID NO 19
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 19

tgcctccggt tctgaaggtg ttcttg 26

<210> SEQ ID NO 20
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 20

ccggttctga aggtgttctt 20

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tccggttctg aaggtgttct t 21

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ctccggttct gaaggtgttc tt 22

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cctccggttc tgaaggtgtt ctt 23

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<400> SEQUENCE: 24

gcctccggtt ctgaaggtgt tctt 24

<210> SEQ ID NO 25
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<400> SEQUENCE: 25

tgcctccggt tctgaaggtg ttctt 25

<210> SEQ ID NO 26
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<220> FEATURE:
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<400> SEQUENCE: 26

ccggttctga aggtgttct 19

<210> SEQ ID NO 27
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 27

tccggttctg aaggtgttct 20

<210> SEQ ID NO 28
<211> LENGTH: 21
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 28

ctccggttct gaaggtgttc t 21

<210> SEQ ID NO 29
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<400> SEQUENCE: 29

cctccggttc tgaaggtgtt ct 22

<210> SEQ ID NO 30
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 30

gcctccggtt ctgaaggtgt tct 23

<210> SEQ ID NO 31

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<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 31

tgcctccggt tctgaaggtg ttct 24

<210> SEQ ID NO 32
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 32

ccggttctga aggtgttc 18

<210> SEQ ID NO 33
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<400> SEQUENCE: 33

tccggttctg aaggtgttc 19

<210> SEQ ID NO 34
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 34

ctccggttct gaaggtgttc 20

<210> SEQ ID NO 35
<211> LENGTH: 21
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<400> SEQUENCE: 35

cctccggttc tgaaggtgtt c 21

<210> SEQ ID NO 36
<211> LENGTH: 22
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 36

gcctccggtt ctgaaggtgt tc 22

<210> SEQ ID NO 37
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 37

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<210> SEQ ID NO 38
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 38

cattcaactg ttgcctccgg ttctgaaggt g                                 31

<210> SEQ ID NO 39
<211> LENGTH: 30
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 39

ttgcctccgg ttctgaaggt gttcttgtac                                   30

<210> SEQ ID NO 40
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<400> SEQUENCE: 40

aggatttgga acagaggcgt c                                            21

<210> SEQ ID NO 41
<211> LENGTH: 20
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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 41

gtctgccact ggcggaggtc                                              20

<210> SEQ ID NO 42
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 42

catcaagcag aaggcaacaa                                              20

<210> SEQ ID NO 43
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 43

gaagtttcag ggccaagtca                                              20

<210> SEQ ID NO 44
<211> LENGTH: 963
<212> TYPE: DNA
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

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tgctcctttg ccacaacgga cgacttctat gacgacccgt gtttcgactc cccggacctg     120
cgcttcttcg aagacctgga cccgcgcctg atgcacgtgg gcgcgctcct gaaacccgaa     180
gagcactcgc acttccccgc ggcggtgcac ccggccccgg gcgcacgtga ggacgagcat     240
gtgcgcgcgc ccagcgggca ccaccaggcg ggccgctgcc tactgtgggc ctgcaaggcg     300
tgcaagcgca agaccaccaa cgccgaccgc cgcaaggccg ccaccatgcg cgagcggcgc     360
cgcctgagca aagtaaatga ggcctttgag acactcaagc gctgcacgtc gagcaatcca     420
aaccagcggt tgcccaaggt ggagatcctg cgcaacgcca tccgctatat cgagggcctg     480
caggctctgc tgcgcgacca ggacgccgcg ccccctggcg ccgcagccgc cttctatgcg     540
ccgggcccgc tgcccccggg ccgcggcggc gagcactaca gcggcgactc cgacgcgtcc     600
agcccgcgct ccaactgctc cgacggcatg atggactaca gcggcccccc gagcggcgcc     660
cggcggcgga actgctacga aggcgcctac tacaacgagg cgcccagcga acccaggccc     720
gggaagagtg cggcggtgtc gagcctagac tgcctgtcca gcatcgtgga gcgcatctcc     780
accgagagcc ctgcggcgcc cgccctcctg ctggcggacg tgccttctga gtcgcctccg     840
cgcaggcaag aggctgccgc ccccagcgag ggagagagca gcggcgaccc cacccagtca     900
ccggacgccg ccccgcagtg ccctgcgggt gcgaacccca acccgatata ccaggtgctc     960
tga                                                                   963
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<210> SEQ ID NO 45
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 45

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cgggcttgga cagaacttac                                                  20
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<210> SEQ ID NO 46
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 46

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tccttacggg tagcatcctg                                                  20
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<210> SEQ ID NO 47
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 47

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ctgaaggtgt tcttgtactt catcc                                            25
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<210> SEQ ID NO 48
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 48

tgttgagaaa tggcggcgt 19

<210> SEQ ID NO 49
<211> LENGTH: 31
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 49

cauucaacug uugccuccgg uucugaaggu g 31

<210> SEQ ID NO 50
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 50

ucccacugau ucugaauucu uucaa 25

<210> SEQ ID NO 51
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 51

cuucauccca cugauucuga auucu 25

<210> SEQ ID NO 52
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 52

uuguacuuca ucccacugau ucuga 25

<210> SEQ ID NO 53
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 53

uguucuugua cuucauccca cugau 25

<210> SEQ ID NO 54
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 54

gaagguguuc uuguacuuca uccca 25

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<210> SEQ ID NO 55
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 55

guucugaagg uguucuugua cuuca 25

<210> SEQ ID NO 56
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 56

cuccgguucu gaagguguuc uugua 25

<210> SEQ ID NO 57
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 57

guugccuccg guucugaagg uguuc 25

<210> SEQ ID NO 58
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 58

caacuguugc cuccgguucu gaagg 25

<210> SEQ ID NO 59
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 59

ucauucaacu guugccuccg guucu 25

<210> SEQ ID NO 60
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 60

acauuucauu caacuguugc cuccg 25

<210> SEQ ID NO 61
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

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<400> SEQUENCE: 61

cuuuaacauu ucauucaacu guugc 25

<210> SEQ ID NO 62
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 62

gaauccuuua acauuucauu caacu 25

<210> SEQ ID NO 63
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 63

guguugaauc cuuuaacauu ucauu 25

<210> SEQ ID NO 64
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 64

ccauuguguu gaauccuuua acauu 25

<210> SEQ ID NO 65
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 65

uccagccauu guguugaauc cuuua 25

<210> SEQ ID NO 66
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 66

uagcuuccag ccauuguguu gaauc 25

<210> SEQ ID NO 67
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 67

uuccuuagcu uccagccauu guguu 25

<210> SEQ ID NO 68

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<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 68

gcuucuuccu uagcuuccag ccauu 25

<210> SEQ ID NO 69
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 69

gcucagcuuc uuccuuagcu uccag 25

<210> SEQ ID NO 70
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 70

gaccugcuca gcuucuuccu uagcu 25

<210> SEQ ID NO 71
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 71

ccuaagaccu gcucagcuuc uuccu 25

<210> SEQ ID NO 72
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 72

ccuguccuaa gaccugcuca gcuuc 25

<210> SEQ ID NO 73
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 73

ucuggccugu ccuaagaccu gcuca 25

<210> SEQ ID NO 74
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 74

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<210> SEQ ID NO 75
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 75

caagcuuggc ucuggccugu ccuaa                                        25


<210> SEQ ID NO 76
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 76

ugacucaagc uuggcucugg ccugu                                        25


<210> SEQ ID NO 77
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 77

uuccaugacu caagcuuggc ucugg                                        25


<210> SEQ ID NO 78
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 78

ccuccuucca ugacucaagc uuggc                                        25


<210> SEQ ID NO 79
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 79

gggacccucc uuccaugacu caagc                                        25


<210> SEQ ID NO 80
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid
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<400> SEQUENCE: 80

guauagggac ccuccuucca ugacu 25

<210> SEQ ID NO 81
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 81

cuacuguaua gggacccucc uucca 25

<210> SEQ ID NO 82
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 82

ugcaucuacu guauagggac ccucc 25

<210> SEQ ID NO 83
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 83

uggauugcau cuacuguaua gggac 25

<210> SEQ ID NO 84
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 84

ucuuuuggau ugcaucuacu guaua 25

<210> SEQ ID NO 85
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 85

gauuuucuuu uggauugcau cuacu 25

<210> SEQ ID NO 86
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 86

ucugugauuu ucuuuuggau ugcau 25

<210> SEQ ID NO 87

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<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 87

ugguuucugu gauuuucuuu uggau                                        25


<210> SEQ ID NO 88
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 88

ccuuagcuuc cagccauugu guuga                                        25


<210> SEQ ID NO 89
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 89

ucuuccuuag cuuccagcca uugug                                        25


<210> SEQ ID NO 90
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 90

ggcucuggcc uguccuaaga ccugc                                        25


<210> SEQ ID NO 91
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 91

agcuuggcuc uggccugucc uaaga                                        25


<210> SEQ ID NO 92
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 92

cucaagcuug gcucuggccu guccu                                        25


<210> SEQ ID NO 93
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 93
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gacccucccu ccaugacuca agcuu                                   25


<210> SEQ ID NO 94
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 94

auagggaccc ucccuuccaug acuca                                  25


<210> SEQ ID NO 95
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 95

cuguauaggg acccucccuuc cauga                                  25


<210> SEQ ID NO 96
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 96

ugugauuuuc uuuuggauug caucu                                   25


<210> SEQ ID NO 97
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 97

guuucuguga uuuucuuuug gauug                                   25


<210> SEQ ID NO 98
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 98

cuugguuucu gugauuuucu uuugg                                   25


<210> SEQ ID NO 99
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 99

ccgguucuga agguguucuu guacu                                   25


<210> SEQ ID NO 100
<211> LENGTH: 25
<212> TYPE: RNA
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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

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The invention claimed is:

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of the nucleotide sequence of SEQ ID NO: 57, wherein the antisense oligomer is an oligonucleotide in which the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified, or a morpholino oligomer.

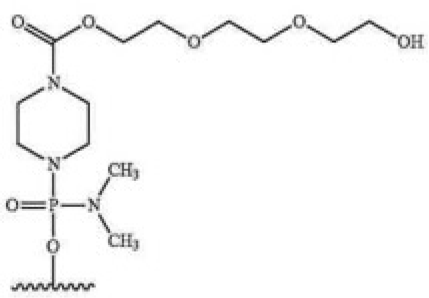
2. The antisense oligomer according to claim 1, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

3. The antisense oligomer according to claim 1, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of: a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond, and a boranophosphate bond.

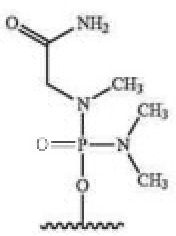
4. The antisense oligomer according to claim 1, which is a morpholino oligomer.

5. The antisense oligomer according to claim 4, which is a phosphorodiamidate morpholino oligomer.

6. The antisense oligomer according to claim 4, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



(2)

-continued

(3)

7. A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to claim 1, or a pharmaceutically acceptable salt or hydrate thereof.

* * * * *

EXHIBIT 11

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(71) Applicant (*for all designated States except US*): **THE UNIVERSITY OF WESTERN AUSTRALIA** [AU/AU]; Stirling Highway, Nedlands, Western Australia 6907 (AU).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **WILTON, Stephen, Donald** [AU/AU]; 18 Spey Road, Applecross, Western Australia 6153 (AU). **FLETCHER, Sue** [AU/AU]; 14 Roberts Street, Bayswater, Western Australia 6053 (AU). **MCCLOREY, Graham** [AU/AU]; 8 Digwood Close, Bayswater, Western Australia 6053 (AU).

(74) Agent: **WRAY & ASSOCIATES**; Level 4, The Quadrant, 1 William Street, Perth, Western Australia 6000 (AU).

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF

(57) Abstract: An antisense molecule capable of binding to a selected target site to induce exon skipping in the dystrophin gene, as set forth in SEQ ID NO: 1 to 202.

"Antisense Oligonucleotides for Inducing Exon Skipping and Methods of Use Thereof"

Field of the Invention

The present invention relates to novel antisense compounds and compositions suitable for facilitating exon skipping. It also provides methods for inducing exon skipping using the novel antisense compounds as well as therapeutic compositions adapted for use in the methods of the invention..

Background Art

Significant effort is currently being expended researching methods for suppressing or compensating for disease-causing mutations in genes. Antisense technologies are being developed using a range of chemistries to affect gene expression at a variety of different levels (transcription, splicing, stability, translation). Much of that research has focused on the use of antisense compounds to correct or compensate for abnormal or disease-associated genes in a myriad of different conditions.

Antisense molecules are able to inhibit gene expression with exquisite specificity and because of this many research efforts concerning oligonucleotides as modulators of gene expression have focused on inhibiting the expression of targeted genes such as oncogenes or viral genes. The antisense oligonucleotides are directed either against RNA (sense strand) or against DNA where they form triplex structures inhibiting transcription by RNA polymerase II. To achieve a desired effect in specific gene down-regulation, the oligonucleotides must either promote the decay of the targeted mRNA or block translation of that mRNA, thereby effectively preventing *de novo* synthesis of the undesirable target protein.

Such techniques are not useful where the object is to up-regulate production of the native protein or compensate for mutations which induce premature termination of translation such as nonsense or frame-shifting mutations. Furthermore, in cases where a normally functional protein is prematurely

terminated because of mutations therein, a means for restoring some functional protein production through antisense technology has been shown to be possible through intervention during the splicing processes (Sierakowska H, *et al.,* (1996) Proc Natl Acad Sci USA 93,12840-12844; Wilton SD, *et al.*, (1999) Neuromusc Disorders 9, 330-338; van Deutekom JC *et al.*, (2001) Human Mol Genet 10, 1547-1554). In these cases, the defective gene transcript should not be subjected to targeted degradation so the antisense oligonucleotide chemistry should not promote target mRNA decay.

In a variety of genetic diseases, the effects of mutations on the eventual expression of a gene can be modulated through a process of targeted exon skipping during the splicing process. The splicing process is directed by complex multi-particle machinery that brings adjacent exon-intron junctions in pre-mRNA into close proximity and performs cleavage of phosphodiester bonds at the ends of the introns with their subsequent reformation between exons that are to be spliced together. This complex and highly precise process is mediated by sequence motifs in the pre-mRNA that are relatively short semi-conserved RNA segments to which bind the various nuclear splicing factors that are then involved in the splicing reactions. By changing the way the splicing machinery reads or recognises the motifs involved in pre-mRNA processing, it is possible to create differentially spliced mRNA molecules. It has now been recognised that the majority of human genes are alternatively spliced during normal gene expression, although the mechanisms invoked have not been identified. Using antisense oligonucleotides, it has been shown that errors and deficiencies in a coded mRNA could be bypassed or removed from the mature gene transcripts.

In nature, the extent of genetic deletion or exon skipping in the splicing process is not fully understood, although many instances have been documented to occur, generally at very low levels (Sherrat TG, *et al.,* (1993) Am J Hum Genet 53, 1007-1015). However, it is recognised that if exons associated with disease-causing mutations can be specifically deleted from some genes, a shortened protein product can sometimes be produced that has similar biological properties of the native protein or has sufficient biological activity to ameliorate the disease caused by mutations associated with the target exon (Lu QL, *et al.*, (2003)

Nature Medicine 9, 1009-1014; Aartsma-Rus A *et al.*, (2004) Am J Hum Genet 74: 83-92).

This process of targeted exon skipping is likely to be particularly useful in long genes where there are many exons and introns, where there is redundancy in the genetic constitution of the exons or where a protein is able to function without one or more particular exons (e.g. with the dystrophin gene, which consists of 79 exons; or possibly some collagen genes which encode for repeated blocks of sequence or the huge nebulin or titin genes which are comprised of ~80 and over 370 exons, respectively).

Efforts to redirect gene processing for the treatment of genetic diseases associated with truncations caused by mutations in various genes have focused on the use of antisense oligonucleotides that either: (1) fully or partially overlap with the elements involved in the splicing process; or (2) bind to the pre-mRNA at a position sufficiently close to the element to disrupt the binding and function of the splicing factors that would normally mediate a particular splicing reaction which occurs at that element (e.g., binds to the pre-mRNA at a position within 3, 6, or 9 nucleotides of the element to be blocked).

For example, modulation of mutant dystrophin pre-mRNA splicing with antisense oligoribonucleotides has been reported both *in vitro* and *in vivo*. In one type of dystrophin mutation reported in Japan, a 52-base pair deletion mutation causes exon 19 to be removed with the flanking introns during the splicing process (Matsuo *et al.*, (1991) J Clin Invest. 87:2127-2131). An *in vitro* minigene splicing system has been used to show that a 31-mer 2'-O-methyl oligoribonucleotide complementary to the 5' half of the deleted sequence in dystrophin Kobe exon 19 inhibited splicing of wild-type *pre-mRNA* (Takeshima *et al.* (1995), J. Clin. Invest., 95, 515-520). The same oligonucleotide was used to induce exon skipping from the native dystrophin gene transcript in human cultured lymphoblastoid cells.

Dunckley *et al.*, (1997) Nucleosides & Nucleotides, 16, 1665-1668 described *in vitro* constructs for analysis of splicing around exon 23 of mutated dystrophin in the *mdx* mouse mutant, a model for muscular dystrophy. Plans to analyse these constructs *in vitro* using 2' modified oligonucleotides targeted to splice sites

within and adjacent to mouse dystrophin exon 23 were discussed, though no target sites or sequences were given.

2'-O-methyl oligoribonucleotides were subsequently reported to correct dystrophin deficiency in myoblasts from the *mdx* mouse from this group. An antisense oligonucleotide targeted to the 3' splice site of murine dystrophin intron 22 was reported to cause skipping of the mutant exon as well as several flanking exons and created a novel in-frame dystrophin transcript with a novel internal deletion. This mutated dystrophin was expressed in 1-2% of antisense treated mdx myotubes. Use of other oligonucleotide modifications such as 2'-O-methoxyethyl phosphodiesters are described (Dunckley *et al*. (1998) Human Mol. Genetics, 5, 1083-90).

Thus, antisense molecules may provide a tool in the treatment of genetic disorders such as Duchenne Muscular Dystrophy (DMD). However, attempts to induce exon skipping using antisense molecules have had mixed success. Studies on dystrophin exon 19, where successful skipping of that exon from the dystrophin pre-mRNA was achieved using a variety of antisense molecules directed at the flanking splice sites or motifs within the exon involved in exon definition as described by Errington *et al*. (2003) J Gen Med 5, 518-527".

In contrast to the apparent ease of exon 19 skipping, the first report of exon 23 skipping in the *mdx* mouse by Dunckley *et al*., (1998) is now considered to be reporting only a naturally occurring revertant transcript or artefact rather than any true antisense activity. In addition to not consistently generating transcripts missing exon 23, Dunckley *et al*., (1998) did not show any time course of induced exon skipping, or even titration of antisense oligonucleotides, to demonstrate dose dependent effects where the levels of exon skipping corresponded with increasing or decreasing amounts of antisense oligonucleotide. Furthermore, this work could not be replicated by other researchers.

The first example of specific and reproducible exon skipping in the *mdx* mouse model was reported by Wilton *et* al., (1999) Neuromuscular Disorders 9, 330-338. By directing an antisense molecule to the donor splice site, consistent and

efficient exon 23 skipping was induced in the dystrophin mRNA within 6 hours of treatment of the cultured cells. Wilton *et al.,* (1999), also describe targeting the acceptor region of the mouse dystrophin pre-mRNA with longer antisense oligonucleotides and being unable to repeat the published results of Dunckley *et al.*, (1998). No exon skipping, either 23 alone or multiple removal of several flanking exons, could be reproducibly detected using a selection of antisense oligonucleotides directed at the acceptor splice site of intron 22.

While the first antisense oligonucleotide directed at the intron 23 donor splice site induced consistent exon skipping in primary cultured myoblasts, this compound was found to be much less efficient in immortalized cell cultures expressing higher levels of dystrophin. However, with refined targeting and antisense oligonucleotide design, the efficiency of specific exon removal was increased by almost an order of magnitude (see Mann CJ *et al.*, (2002) J Gen Med 4, 644-654).

Thus, there remains a need to provide antisense oligonucleotides capable of binding to and modifying the splicing of a target nucleotide sequence. Simply directing the antisense oligonucleotides to motifs presumed to be crucial for splicing is no guarantee of the efficacy of that compound in a therapeutic setting.

Summary of the Invention

The present invention provides antisense molecule compounds and compositions suitable for binding to RNA motifs involved in the splicing of pre-mRNA that are able to induce specific and efficient exon skipping and a method for their use thereof.

The choice of target selection plays a crucial role in the efficiency of exon skipping and hence its subsequent application of a potential therapy. Simply designing antisense molecules to target regions of pre-mRNA presumed to be involved in splicing is no guarantee of inducing efficient and specific exon skipping. The most obvious or readily defined targets for splicing intervention are the donor and acceptor splice sites although there are less defined or conserved motifs including exonic splicing enhancers, silencing elements and branch points.

The acceptor and donor splice sites have consensus sequences of about 16 and 8 bases respectively (see Figure 1 for schematic representation of motifs and domains involved in exon recognition, intron removal and the splicing process).

According to a first aspect, the invention provides antisense molecules capable of binding to a selected target to induce exon skipping.

For example, to induce exon skipping in exons 3 to 8, 10 to 16, 19 to 40, 42 to 44, 46, 47, and 50 to 53 in the Dystrophin gene transcript the antisense molecules are preferably selected from the group listed in Table 1A.

In a further example, it is possible to combine two or more antisense oligonucleotides of the present invention together to induce multiple exon skipping in exons 19-20, and 53. This is a similar concept to targeting of a single exon. A combination or "cocktail" of antisense oligonucleotides are directed at adjacent exons to induce efficient exon skipping.

In another example, to induce exon skipping in exons 19-20, 31, 34 and 53 it is possible to improve exon skipping of a single exon by joining together two or more antisense oligonucleotide molecules. This concept is termed by the inventor as a "weasel", an example of a cunningly designed antisense oligonucleotide. A similar concept has been described in Aartsma-Rus A *et al.*, (2004) Am J Hum Genet 74: 83-92).

According to a second aspect, the present invention provides antisense molecules selected and or adapted to aid in the prophylactic or therapeutic treatment of a genetic disorder comprising at least an antisense molecule in a form suitable for delivery to a patient.

According to a third aspect, the invention provides a method for treating a patient suffering from a genetic disease wherein there is a mutation in a gene encoding a particular protein and the affect of the mutation can be abrogated by exon skipping, comprising the steps of: (a) selecting an antisense molecule in accordance with the methods described herein; and (b) administering the molecule to a patient in need of such treatment.

The invention also addresses the use of purified and isolated antisense oligonucleotides of the invention, for the manufacture of a medicament for treatment of a genetic disease.

The invention further provides a method of treating a condition characterised by Duchenne muscular dystrophy, which method comprises administering to a patient in need of treatment an effective amount of an appropriately designed antisense oligonucleotide of the invention, relevant to the particular genetic lesion in that patient. Further, the invention provides a method for prophylactically treating a patient to prevent or at least minimise Duchene muscular dystrophy, comprising the step of: administering to the patient an effective amount of an antisense oligonucleotide or a pharmaceutical composition comprising one or more of these biological molecules.

The invention also provides kits for treating a genetic disease, which kits comprise at least a antisense oligonucleotide of the present invention, packaged in a suitable container and instructions for its use.

Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following figures.

Brief Description of the Drawings

Figure 1 Schematic representation of motifs and domains involved in exon recognition, intron removal and the splicing process.

Figure 2. Diagrammatic representation of the concept of antisense oligonucleotide induced exon skipping to by-pass disease-causing mutations (not drawn to scale). The hatched box represents an exon carrying a mutation that prevents the translation of the rest of the mRNA into a protein. The solid black bar represents an antisense oligonucleotide that prevents inclusion of that exon in the mature mRNA.

Figure 3 Gel electrophoresis showing differing efficiencies of two antisense molecules directed at exon 8 acceptor splice site. The preferred compound [H8A(-06+18)] induces strong and consistent exon skipping at a transfection concentration of 20 nanomolar in cultured normal human muscle cells. The less preferred antisense oligonucleotide [H8A(-06+14)] also induces efficient exon skipping, but at much higher concentrations. Other antisense oligonucleotides directed at exon 8 either only induced lower levels of exon skipping or no detectable skipping at all (not shown).

Figure 4 Gel electrophoresis showing differing efficiencies of two antisense molecules directed at internal domains within exon 7, presumably exon splicing enhancers. The preferred compound [H7A(+45+67)] induces strong and consistent exon skipping at a transfection concentration of 20 nanomolar in cultured human muscle cells. The less preferred antisense oligonucleotide [H7A(+2+26)] induces only low levels of exon skipping at the higher transfection concentrations. Other antisense oligonucleotides directed at exon 7 either only induced lower levels of exon skipping or no detectable skipping at all (not shown).

Figure 5 Gel electrophoresis showing an example of low efficiency exon 6 skipping using two non-preferred antisense molecules directed at human exon 6 donor splice site. Levels of induced exon 6 skipping are either very low [H6D(+04-21)] or almost undetectable [H6D(+18-04)]. These are examples of non-preferred antisense oligonucleotides to demonstrate that antisense oligonucleotide design plays a crucial role in the efficacy of these compounds.

Figure 6 Gel electrophoresis showing strong and efficient human exon 6 skipping using an antisense molecules [H6A(+69+91)] directed at an exon 6 internal domain, presumably an exon splicing enhancer. This preferred compound induces consistent exon skipping at a

transfection concentration of 20 nanomolar in cultured human muscle cells.

Figure 7 Gel electrophoresis showing strong human exon 4 skipping using an antisense molecule H4A(+13+32) directed at an exon 6 internal domain, presumably an exon splicing enhancer. This preferred compound induces strong and consistent exon skipping at a transfection concentration of 20 nanomolar in cultured human muscle cells.

Figure 8 Gel electrophoresis showing (8B) strong human exon 11 skipping using antisense molecule H11A(+75+97) directed at an exon 11 internal domain; and (8B) strong human exon 12 skipping using antisense molecule H12A(+52+75) directed at exon 12 internal domain.

Figure 9 Gel electrophoresis showing (9A) strong human exon 15 skipping using antisense molecules H15A(+48+71) and H15A(-12+19) directed at an exon 15 internal domain; and (9B) strong human exon 16 skipping using antisense molecules H16A(-12+19) and H16A(-06+25).

Figure 10 Gel electrophoresis showing human exon 19/20 skipping using antisense molecules H20A(+44+71) and H20A(+149+170) directed at an exon 20 and a “cocktail” of antisense oligonucleotides H19A(+35+65, H20A(+44+71) and H20A(+149+170) directed at exons 19/20.

Figure 11 Gel electrophoresis showing human exon 19/20 skipping using “weasels” directed at exons 19 and 20.

Figure 12 Gel electrophoresis showing exon 22 skipping using antisense molecules H22A(+125+106), H22A(+47+69), H22A(+80+101) and H22D(+13-11) directed at exon 22.

Figure 13 Gel electrophoresis showing exon 31 skipping using antisense molecules H31D(+01-25) and H31D(+03-22); and a "cocktail" of antisense molecules directed at exon 31.

Figure 14 Gel electrophoresis showing exon 33 skipping using antisense molecules H33A(+30+56) and H33A(+64+88) directed at exon 33.

Figure 15 Gel electrophoresis showing exon 35 skipping using antisense molecules H35A(+141+161), H35A(+116+135), and H35A(+24+43) and a "cocktail of two antisense molecules, directed at exon 35.

Figure 16 Gel electrophoresis showing exon 36 skipping using antisense molecules H32A(+49+73) and H36A(+26+50) directed at exon 36.

Figure 17 Gel electrophoresis showing exon 37 skipping using antisense molecules H37A(+82+105) and H37A(+134+157) directed at exon 37.

Figure 18 Gel electrophoresis showing exon 38 skipping using antisense molecule H38A(+88+112) directed at exon 38.

Figure 19 Gel electrophoresis showing exon 40 skipping using antisense molecule H40A(-05+17) directed at exon 40.

Figure 20 Gel electrophoresis showing exon 42 skipping using antisense molecule H42A(-04+23) directed at exon 42.

Figure 21 Gel electrophoresis showing exon 46 skipping using antisense molecule H46A(+86+115) directed at exon 46

Figure 22 Gel electrophoresis showing exon 51, exon 52 and exon 53 skipping using various antisense molecules directed at exons 51, 52 and 53, respectively. A "cocktail" of antisense molecules is also shown directed at exon 53.

Brief Description of the Sequence listings

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
1	H8A(-06+18)	GAU AGG UGG UAU CAA CAU CUG UAA
2	H8A (-03+18)	GAU AGG UGG UAU CAA CAU CUG
3	H8A(-07+18)	GAU AGG UGG UAU CAA CAU CUG UAA G
4	H8A(-06+14)	GGU GGU AUC AAC AUC UGU AA
5	H8A(-10+10)	GUA UCA ACA UCU GUA AGC AC
6	H7A(+45+67)	UGC AUG UUC CAG UCG UUG UGU GG
7	H7A(+02+26)	CAC UAU UCC AGU CAA AUA GGU CUG G
8	H7D(+15-10)	AUU UAC CAA CCU UCA GGA UCG AGU A
9	H7A(-18+03)	GGC CUA AAA CAC AUA CAC AUA
10	C6A(-10+10)	CAU UUU UGA CCU ACA UGU GG
11	C6A(-14+06)	UUU GAC CUA CAU GUG GAA AG
12	C6A(-14+12)	UAC AUU UUU GAC CUA CAU GUG GAA AG
13	C6A(-13+09)	AUU UUU GAC CUA CAU GGG AAA G
14	CH6A(+69+91)	UAC GAG UUG AUU GUC GGA CCC AG
15	C6D(+12-13)	GUG GUC UCC UUA CCU AUG ACU GUG G
16	C6D(+06-11)	GGU CUC CUU ACC UAU GA
17	H6D(+04-21)	UGU CUC AGU AAU CUU CUU ACC UAU
18	H6D(+18-04)	UCU UAC CUA UGA CUA UGG AUG AGA
19	H4A(+13+32)	GCA UGA ACU CUU GUG GAU CC
20	H4D(+04-16)	CCA GGG UAC UAC UUA CAU UA
21	H4D(-24-44)	AUC GUG UGU CAC AGC AUC CAG
22	H4A(+11+40)	UGU UCA GGG CAU GAA CUC UUG UGG AUC CUU
23	H3A(+30+60)	UAG GAG GCG CCU CCC AUC CUG UAG GUC ACU G
24	H3A(+35+65)	AGG UCU AGG AGG CGC CUC CCA UCC UGU AGG U
25	H3A(+30+54)	GCG CCU CCC AUC CUG UAG GUC ACU G
26	H3D(+46-21)	CUU CGA GGA GGU CUA GGA GGC GCC UC
27	H3A(+30+50)	CUC CCA UCC UGU AGG UCA CUG
28	H3D(+19-03)	UAC CAG UUU UUG CCC UGU CAG G
29	H3A(-06+20)	UCA AUA UGC UGC UUC CCA AAC UGA AA
30	H3A(+37+61)	CUA GGA GGC GCC UCC CAU CCU GUA G
31	H5A(+20+50)	UUA UGA UUU CCA UCU ACG AUG UCA GUA CUU C
32	H5D(+25-05)	CUU ACC UGC CAG UGG AGG AUU AUA UUC CAA A
33	H5D(+10-15)	CAU CAG GAU UCU UAC CUG CCA GUG G

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
34	H5A(+10+34)	CGA UGU CAG UAC UUC CAA UAU UCA C
35	H5D(-04-21)	ACC AUU CAU CAG GAU UCU
36	H5D(+16-02)	ACC UGC CAG UGG AGG AUU
37	H5A(-07+20)	CCA AUA UUC ACU AAA UCA ACC UGU UAA
38	H5D(+18-12)	CAG GAU UCU UAC CUG CCA GUG GAG GAU UAU
39	H5A(+05+35)	ACG AUG UCA GUA CUU CCA AUA UUC ACU AAA U
40	H5A(+15+45)	AUU UCC AUC UAC GAU GUC AGU ACU UCC AAU A
41	H10A(-05+16)	CAG GAG CUU CCA AAU GCU GCA
42	H10A(-05+24)	CUU GUC UUC AGG AGC UUC CAA AUG CUG CA
43	H10A(+98+119)	UCC UCA GCA GAA AGA AGC CAC G
44	H10A(+130+149)	UUA GAA AUC UCU CCU UGU GC
45	H10A(-33-14)	UAA AUU GGG UGU UAC ACA AU
46	H11D(+26+49)	CCC UGA GGC AUU CCC AUC UUG AAU
47	H11D(+11-09)	AGG ACU UAC UUG CUU UGU UU
48	H11A(+118+140)	CUU GAA UUU AGG AGA UUC AUC UG
49	H11A(+75+97)	CAU CUU CUG AUA AUU UUC CUG UU
50	H12A(+52+75)	UCU UCU GUU UUU GUU AGC CAG UCA
51	H12A(-10+10)	UCU AUG UAA ACU GAA AAU UU
52	H12A(+11+30)	UUC UGG AGA UCC AUU AAA AC
53	H13A(+77+100)	CAG CAG UUG CGU GAU CUC CAC UAG
54	H13A(+55+75)	UUC AUC AAC UAC CAC CAC CAU
55	H13D(+06-19)	CUA AGC AAA AUA AUC UGA CCU UAA G
56	H14A(+37+64)	CUU GUA AAA GAA CCC AGC GGU CUU CUG U
57	H14A(+14+35)	CAU CUA CAG AUG UUU GCC CAU C
58	H14A(+51+73)	GAA GGA UGU CUU GUA AAA GAA CC
59	H14D(-02+18)	ACC UGU UCU UCA GUA AGA CG
60	H14D(+14-10)	CAU GAC ACA CCU GUU CUU CAG UAA
61	H14A(+61+80)	CAU UUG AGA AGG AUG UCU UG
62	H14A(-12+12)	AUC UCC CAA UAC CUG GAG AAG AGA
63	H15A(-12+19)	GCC AUG CAC UAA AAA GGC ACU GCA AGA CAU U
64	H15A(+48+71)	UCU UUA AAG CCA GUU GUG UGA AUC
65	H15A(+08+28)	UUU CUG AAA GCC AUG CAC UAA
66	H15D(+17-08)	GUA CAU ACG GCC AGU UUU UGA AGA C
67	H16A(-12+19)	CUA GAU CCG CUU UUA AAA CCU GUU AAA ACA A
68	H16A(-06+25)	UCU UUU CUA GAU CCG CUU UUA AAA CCU [illegible]

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
		GUU A
69	H16A(-06+19)	CUA GAU CCG CUU UUA AAA CCU GUU A
70	H16A(+87+109)	CCG UCU UCU GGG UCA CUG ACU UA
71	H16A(-07+19)	CUA GAU CCG CUU UUA AAA CCU GUU AA
72	H16A(-07+13)	CCG CUU UUA AAA CCU GUU AA
73	H16A(+12+37)	UGG AUU GCU UUU UCU UUU CUA GAU CC
74	H16A(+92+116)	CAU GCU UCC GUC UUC UGG GUC ACU G
75	H16A(+45+67)	G AUC UUG UUU GAG UGA AUA CAG U
76	H16A(+105+126)	GUU AUC CAG CCA UGC UUC CGU C
77	H16D(+05-20)	UGA UAA UUG GUA UCA CUA ACC UGU G
78	H16D(+12-11)	GUA UCA CUA ACC UGU GCU GUA C
79	H19A(+35+53)	CAG CAG UAG UUG UCA UCU GC
80	H19A(+35+65)	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U
81	H20A(+44+71)	CUG GCA GAA UUC GAU CCA CCG GCU GUU C
82	H20A(+149+170)	CAG CAG UAG UUG UCA UCU GCU C
83	H20A(+185+203)	UGA UGG GGU GGU GGG UUG G
84	H20A(-08+17)	AUC UGC AUU AAC ACC CUC UAG AAA G
85	H20A(+30+53)	CCG GCU GUU CAG UUG UUC UGA GGC
86	H20A(-11+17)	AUC UGC AUU AAC ACC CUC UAG AAA GAA A
87	H20D(+08-20)	GAA GGA GAA GAG AUU CUU ACC UUA CAA A
88	H20A(+44+63)	AUU CGA UCC ACC GGC UGU UC
89	H20A(+149+168	CUG CUG GCA UCU UGC AGU U
90	H21A(-06+16)	GCC GGU UGA CUU CAU CCU GUG C
91	H21A(+85+106)	CUG CAU CCA GGA ACA UGG GUC C
92	H21A(+85+108)	GUC UGC AUC CAG GAA CAU GGG UC
93	H21A(+08+31)	GUU GAA GAU CUG AUA GCC GGU UGA
94	H21D(+18-07)	UAC UUA CUG UCU GUA GCU CUU UCU
95	H22A(+22+45)	CAC UCA UGG UCU CCU GAU AGC GCA
96	H22A(+125+106)	CUG CAA UUC CCC GAG UCU CUG C
97	H22A(+47+69)	ACU GCU GGA CCC AUG UCC UGA UG
98	H22A(+80+101)	CUA AGU UGA GGU AUG GAG AGU
99	H22D(+13-11)	UAU UCA CAG ACC UGC AAU UCC CC
100	H23A(+34+59)	ACA GUG GUG CUG AGA UAG UAU AGG CC
101	H23A(+18+39)	UAG GCC ACU UUG UUG CUC UUG C
102	H23A(+72+90)	UUC AGA GGG CGC UUU CUU C
103	H24A(+48+70)	GGG CAG GCC AUU CCU CCU UCA GA
104	H24A(-02+22)	UCU UCA GGG UUU GUA UGU GAU UCU
105	H25A(+9+36)	CTG GGC UGA AUU GUC UGA AUA UCA CUG

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
106	H25A(+131+156)	CUG UUG GCA CAU GUG AUC CCA CUG AG
107	H25D(+16-08)	GUC UAU ACC UGU UGG CAC AUG UGA
108	H26A(+132+156)	UGC UUU CUG UAA UUC AUC UGG AGU U
109	H26A(-07+19)	CCU CCU UUC UGG CAU AGA CCU UCC AC
110	H26A(+68+92)	UGU GUC AUC CAU UCG UGC AUC UCU G
111	H27A(+82+106)	UUA AGG CCU CUU GUG CUA CAG GUG G
112	H27A(-4+19)	GGG CCU CUU CUU UAG CUC UCU GA
113	H27D(+19-03)	GAC UUC CAA AGU CUU GCA UUU C
114	H28A(-05+19)	GCC AAC AUG CCC AAA CUU CCU AAG
115	H28A(+99+124)	CAG AGA UUU CCU CAG CUC CGC CAG GA
116	H28D(+16-05)	CUU ACA UCU AGC ACC UCA GAG
117	H29A(+57+81)	UCC GCC AUC UGU UAG GGU CUG UGC C
118	H29A(+18+42)	AUU UGG GUU AUC CUC UGA AUG UCG C
119	H29D(+17-05)	CAU ACC UCU UCA UGU AGU UCU C
120	H30A(+122+147)	CAU UUG AGC UGC GUC CAC CUU GUC UG
121	H30A(+25+50)	UCC UGG GCA GAC UGG AUG CUC UGU UC
122	H30D(+19-04)	UUG CCU GGG CUU CCU GAG GCA UU
123	H31D(+06-18)	UUC UGA AAU AAC AUA UAC CUG UGC
124	H31D(+03-22)	UAG UUU CUG AAA UAA CAU AUA CCU G
125	H31A(+05+25)	GAC UUG UCA AAU CAG AUU GGA
126	H31D(+04-20)	GUU UCU GAA AUA ACA UAU ACC UGU
127	H32D(+04-16)	CAC CAG AAA UAC AUA CCA CA
128	H32A(+151+170)	CAA UGA UUU AGC UGU GAC UG
129	H32A(+10+32)	CGA AAC UUC AUG GAG ACA UCU UG
130	H32A(+49+73)	CUU GUA GAC GCU GCU CAA AAU UGG C
131	H33D(+09-11)	CAU GCA CAC ACC UUU GCU CC
132	H33A(+53+76)	UCU GUA CAA UCU GAC GUC CAG UCU
133	H33A(+30+56)	GUC UUU AUC ACC AUU UCC ACU UCA GAC
134	H33A(+64+88)	CCG UCU GCU UUU UCU GUA CAA UCU G
135	H34A(+83+104)	UCC AUA UCU GUA GCU GCC AGC C
136	H34A(+143+165)	CCA GGC AAC UUC AGA AUC CAA AU
137	H34A(-20+10)	UUU CUG UUA CCU GAA AAG AAU UAU AAU GAA
138	H34A(+46+70)	CAU UCA UUU CCU UUC GCA UCU UAC G
139	H34A(+95+120)	UGA UCU CUU UGU CAA UUC CAU AUC UG
140	H34D(+10-20)	UUC AGU GAU AUA GGU UUU ACC UUU CCC CAG
141	H34A(+72+96)	CUG UAG CUG CCA GCC AUU CUG UCA AG
142	H35A(+141+161)	UCU UCU GCU CGG GAG GUG ACA
143	H35A(+116+135)	CCA GUU ACU AUU CAG AAG AC

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
144	H35A(+24+43)	UCU UCA GGU GCA CCU UCU GU
145	H36A(+26+50)	UGU GAU GUG GUC CAC AUU CUG GUC A
146	H36A(-02+18)	CCA UGU GUU UCU GGU AUU CC
147	H37A(+26+50)	CGU GUA GAG UCC ACC UUU GGG CGU A
148	H37A(+82+105)	UAC UAA UUU CCU GCA GUG GUC ACC
149	H37A(+134+157)	UUC UGU GUG AAA UGG CUG CAA AUC
150	H38A(-01+19)	CCU UCA AAG GAA UGG AGG CC
151	H38A(+59+83)	UGC UGA AUU UCA GCC UCC AGU GGU U
152	H38A(+88+112)	UGA AGU CUU CCU CUU UCA GAU UCA C
153	H39A(+62+85)	CUG GCU UUC UCU CAU CUG UGA UUC
154	H39A(+39+58)	GUU GUA AGU UGU CUC CUC UU
155	H39A(+102+121)	UUG UCU GUA ACA GCU GCU GU
156	H39D(+10-10)	GCU CUA AUA CCU UGA GAG CA
157	H40A(-05+17)	CUU UGA GAC CUC AAA UCC UGU U
158	H40A(+129+153)	CUU UAU UUU CCU UUC AUC UCU GGG C
159	H42A(-04+23)	AUC GUU UCU UCA CGG ACA GUG UGC UGG
160	H42A(+86+109)	GGG CUU GUG AGA CAU GAG UGA UUU
161	H42D(+19-02)	A CCU UCA GAG GAC UCC UCU UGC
162	H43D(+10-15)	UAU GUG UUA CCU ACC CUU GUC GGU C
163	H43A(+101+120)	GGA GAG AGC UUC CUG UAG CU
164	H43A(+78+100)	UCA CCC UUU CCA CAG GCG UUG CA
165	H44A(+85+104)	UUU GUG UCU UUC UGA GAA AC
166	H44D(+10-10)	AAA GAC UUA CCU UAA GAU AC
167	H44A(-06+14)	AUC UGU CAA AUC GCC UGC AG
168	H46D(+16-04)	UUA CCU UGA CUU GCU CAA GC
169	H46A(+90+109)	UCC AGG UUC AAG UGG GAU AC
170	H47A(+76+100)	GCU CUU CUG GGC UUA UGG GAG CAC U
171	H47D(+25-02)	ACC UUU AUC CAC UGG AGA UUU GUC UGC
172	H47A(-9+12)	UUC CAC CAG UAA CUG AAA CAG
173	H50A(+02+30)	CCA CUC AGA GCU CAG AUC UUC UAA CUU CC
174	H50A(+07+33)	CUU CCA CUC AGA GCU CAG AUC UUC UAA
175	H50D(+07-18)	GGG AUC CAG UAU ACU UAC AGG CUC C
176	H51A(-01+25)	ACC AGA GUA ACA GUC UGA GUA GGA GC
177	H51D(+16-07)	CUC AUA CCU UCU GCU UGA UGA UC
178	H51A(+111+134)	UUC UGU CCA AGC CCG GUU GAA AUC
179	H51A(+61+90)	ACA UCA AGG AAG AUG GCA UUU CUA GUU UGG
180	H51A(+66+90)	ACA UCA AGG AAG AUG GCA UUU CUA G
181	H51A(+66+95)	CUC CAA CAU CAA GGA AGA UGG CAU UUC

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
		UAG
182	H51D(+08-17)	AUC AUU UUU UCU CAU ACC UUC UGC U
183	H51A/D(+08-17) & (-15+)	AUC AUU UUU UCU CAU ACC UUC UGC UAG GAG CUA AAA
184	H51A(+175+195)	CAC CCA CCA UCA CCC UCU GUG
185	H51A(+199+220)	AUC AUC UCG UUG AUA UCC UCA A
186	H52A(-07+14)	UCC UGC AUU GUU GCC UGU AAG
187	H52A(+12+41)	UCC AAC UGG GGA CGC CUC UGU UCC AAA UCC
188	H52A(+17+37)	ACU GGG GAC GCC UCU GUU CCA
189	H52A(+93+112)	CCG UAA UGA UUG UUC UAG CC
190	H52D(+05-15)	UGU UAA AAA ACU UAC UUC GA
191	H53A(+45+69)	CAU UCA ACU GUU GCC UCC GGU UCU G
192	H53A(+39+62)	CUG UUG CCU CCG GUU CUG AAG GUG
193	H53A(+39+69)	CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G
194	H53D(+14-07)	UAC UAA CCU UGG UUU CUG UGA
195	H53A(+23+47)	CUG AAG GUG UUC UUG UAC UUC AUC C
196	H53A(+150+176)	UGU AUA GGG ACC CUC CUU CCA UGA CUC
197	H53D(+20-05)	CUA ACC UUG GUU UCU GUG AUU UUC U
198	H53D(+09-18)	GGU AUC UUU GAU ACU AAC CUU GGU UUC
199	H53A(-12+10)	AUU CUU UCA ACU AGA AUA AAA G
200	H53A(-07+18)	GAU UCU GAA UUC UUU CAA CUA GAA U
201	H53A(+07+26)	AUC CCA CUG AUU CUG AAU UC
202	H53A(+124+145)	UUG GCU CUG GCC UGU CCU AAG A
203	H46A(+86+115)	CUC UUU UCC AGG UUC AAG UGG GAU ACU AGC
204	H46A(+107+137)	CAA GCU UUU CUU UUA GUU GCU GCU CUU UUC C
205	H46A(-10+20)	UAU UCU UUU GUU CUU CUA GCC UGG AGA AAG
206	H46A(+50+77)	CUG CUU CCU CCA ACC AUA AAA CAA AUU C
207	H45A(-06+20)	CCA AUG CCA UCC UGG AGU UCC UGU AA
208	H45A(+91 +110)	UCC UGU AGA AUA CUG GCA UC
209	H45A(+125+151)	UGC AGA CCU CCU GCC ACC GCA GAU UCA
210	H45D(+16 -04)	CUA CCU CUU UUU UCU GUC UG
211	H45A(+71+90)	UGU UUU UGA GGA UUG CUG AA

Table 1A: Description of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping

during the processing of the dystrophin pre-mRNA. Since these 2'-O-methyl antisense oligonucleotides are more RNA-like, U represents uracil. With other antisense chemistries such as peptide nucleic acids or morpholinos, these U bases may be shown as "T".

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5'-3')
81 82	H20A(+44+71) H20A(+149+170)	CUG GCA GAA UUC GAU CCA CCG GCU GUU C CAG CAG UAG UUG UCA UCU GCU C
79 81 82	H19A(+35+65) H20A(+44+71) H20A(+149+170)	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U CUG GCA GAA UUC GAU CCA CCG GCU GUU C CAG CAG UAG UUG UCA UCU GCU C
194 195 196	H53D(+14-07) H53A(+23+47) H53A(+150+175)	UAC UAA CCU UGG UUU CUG UGA CTG AAG GUG UUC UUG UAC UUC AUC C UGU AUA GGG ACC CUC CUU CCA UGA CUC

Table 1B: Description of a cocktail of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA.

SEQ ID	SEQUENCE	NUCELOTIDE SEQUENCE (5'-3')
80 82	H20A(+44+71)- H20A(+149+170)	CUG GCA GAA UUC GAU CCA CCG GCU GUU C- CAG CAG UAG UUG UCA UCU GCU C
81 88 89	H19A(+35+53)- H20A(+44+63)- H20A(+149+168)	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U -AUU CGA UCU ACC GGC UGU UC- AA CUG CUG GCA UCU UGC AGU U
80 88	H19A(+35+53)- H20A(+44+63)	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U -AUU CGA UCU ACC GGC UGU UC-
80 89	H19A(+35+53)- H20A(+149+168)	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U -AA CUG CUG GCA UCU UGC AGU U
138 139	H34A(+46+70)- H34A(+94+120)	CAU UCA UUU CCU UUC GCA UCU UAC G- UGA UCU CUU UGU CAA UUC CAU AUC UG
124 144	H31D(+03-22)- UU- H35A(+24+43)	UAG UUU CUG AAA UAA CAU AUA CCU G- UU- UCU UCA GGU GCA CCU UCU GU
195 196	H53A(+23+47)- AA- H53A(+150+175)- AA- H53D(+14-07)	CUG AAG GUG UUC UUG UAC UUC AUC C- UGU AUA GGG ACC CUC CUU CCA UGA CUC- AA- UAC UAA CCU UGG UUU CUG UGA

SEQ ID	SEQUENCE	NUCELOTIDE SEQUENCE (5'-3')
194		
-	Aimed at exons 19/20/20	CAG CAG UAG UUG UCA UCU GCU CAA CUG GCA GAA UUC GAU CCA CCG GCU GUU CAA GCC UGA GCU GAU CUG CUC GCA UCU UGC AGU

Table 1C: Description of a "weasel" of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA.

Detailed Description of the Invention

General

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variation and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

Sequence identity numbers (SEQ ID NO:) containing nucleotide and amino acid sequence information included in this specification are collected at the end of the description and have been prepared using the programme PatentIn Version 3.0. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc.). The length, type of sequence and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the

information provided in numeric indicator field <400> followed by the sequence identifier (e.g. <400>1, <400>2, etc.).

An antisense molecules nomenclature system was proposed and published to distinguish between the different antisense molecules (see Mann *et al*., (2002) J Gen Med 4, 644-654). This nomenclature became especially relevant when testing several slightly different antisense molecules, all directed at the same target region, as shown below:

H # A/D (x : y).

The first letter designates the species (e.g. H: human, M: murine, C: canine)

"#" designates target dystrophin exon number.

"A/D" indicates acceptor or donor splice site at the beginning and end of the exon, respectively.

(x y) represents the annealing coordinates where "-" or "+" indicate intronic or exonic sequences respectively. As an example, A(-6+18) would indicate the last 6 bases of the intron preceding the target exon and the first 18 bases of the target exon. The closest splice site would be the acceptor so these coordinates would be preceded with an "A". Describing annealing coordinates at the donor splice site could be D(+2-18) where the last 2 exonic bases and the first 18 intronic bases correspond to the annealing site of the antisense molecule. Entirely exonic annealing coordinates that would be represented by A(+65+85), that is the site between the 65^{th} and 85^{th} nucleotide from the start of that exon.

The entire disclosures of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference. No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working in the field to which this invention relates.

As used necessarily herein the term "derived" and "derived from" shall be taken to indicate that a specific integer may be obtained from a particular source *albeit* not directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

Description of the Preferred Embodiment

When antisense molecule(s) are targeted to nucleotide sequences involved in splicing in exons within pre-mRNA sequences, normal splicing of the exon may be inhibited causing the splicing machinery to by-pass the entire mutated exon from the mature mRNA. The concept of antisense oligonucleotide induced exon skipping is shown in Figure 2. In many genes, deletion of an entire exon would lead to the production of a non-functional protein through the loss of important functional domains or the disruption of the reading frame. In some proteins, however, it is possible to shorten the protein by deleting one or more exons, without disrupting the reading frame, from within the protein without seriously altering the biological activity of the protein. Typically, such proteins have a structural role and or possess functional domains at their ends. The present invention describes antisense molecules capable of binding to specified dystrophin pre-mRNA targets and re-directing processing of that gene.

Antisense Molecules

According to a first aspect of the invention, there is provided antisense molecules capable of binding to a selected target to induce exon skipping. To induce exon skipping in exons of the Dystrophin gene transcript, the antisense molecules are

preferably selected from the group of compounds shown in Table 1A. There is also provided a combination or "cocktail" of two or more antisense oligonucleotides capable of binding to a selected target to induce exon skipping. To induce exon skipping in exons of the Dystrophin gene transcript, the antisense molecules in a "cocktail" are preferably selected from the group of compounds shown in Table 1B. Alternatively, exon skipping may be induced by antisense oligonucleotides joined together "weasels" preferably selected from the group of compounds shown in Table 1C.

Designing antisense molecules to completely mask consensus splice sites may not necessarily generate any skipping of the targeted exon. Furthermore, the inventors have discovered that size or length of the antisense oligonucleotide itself is not always a primary factor when designing antisense molecules. With some targets such as exon 19, antisense oligonucleotides as short as 12 bases were able to induce exon skipping, albeit not as efficiently as longer (20-31 bases) oligonucleotides. In some other targets, such as murine dystrophin exon 23, antisense oligonucleotides only 17 residues long were able to induce more efficient skipping than another overlapping compound of 25 nucleotides.

The inventors have also discovered that there does not appear to be any standard motif that can be blocked or masked by antisense molecules to redirect splicing. In some exons, such as mouse dystrophin exon 23, the donor splice site was the most amenable to target to re-direct skipping of that exon. It should be noted that designing and testing a series of exon 23 specific antisense molecules to anneal to overlapping regions of the donor splice site showed considerable variation in the efficacy of induced exon skipping. As reported in Mann et al., (2002) there was a significant variation in the efficiency of bypassing the nonsense mutation depending upon antisense oligonucleotide annealing ("Improved antisense oligonucleotide induced exon skipping in the *mdx* mouse model of muscular dystrophy". J Gen Med 4: 644-654). Targeting the acceptor site of exon 23 or several internal domains was not found to induce any consistent exon 23 skipping.

In other exons targeted for removal, masking the donor splice site did not induce any exon skipping. However, by directing antisense molecules to the acceptor

splice site (human exon 8 as discussed below), strong and sustained exon skipping was induced. It should be noted that removal of human exon 8 was tightly linked with the co-removal of exon 9. There is no strong sequence homology between the exon 8 antisense oligonucleotides and corresponding regions of exon 9 so it does not appear to be a matter of cross reaction. Rather the splicing of these two exons is inextricably linked. This is not an isolated instance as the same effect is observed in canine cells where targeting exon 8 for removal also resulted in the skipping of exon 9. Targeting exon 23 for removal in the mouse dystrophin pre-mRNA also results in the frequent removal of exon 22 as well. This effect occurs in a dose dependent manner and also indicates close coordinated processing of 2 adjacent exons.

In other targeted exons, antisense molecules directed at the donor or acceptor splice sites did not induce exon skipping while annealing antisense molecules to intra-exonic regions (i.e. exon splicing enhancers within human dystrophin exon 6) was most efficient at inducing exon skipping. Some exons, both mouse and human exon 19 for example, are readily skipped by targeting antisense molecules to a variety of motifs. That is, targeted exon skipping is induced after using antisense oligonucleotides to mask donor and acceptor splice sites or exon splicing enhancers.

To identify and select antisense oligonucleotides suitable for use in the modulation of exon skipping, a nucleic acid sequence whose function is to be modulated must first be identified. This may be, for example, a gene (or mRNA transcribed form the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (i.e. splice donor sites, splice acceptor sites, or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

Preferably, the present invention aims to provide antisense molecules capable of binding to a selected target in the dystrophin pre-mRNA to induce efficient and consistent exon skipping. Duchenne muscular dystrophy arises from mutations

that preclude the synthesis of a functional dystrophin gene product. These Duchenne muscular dystrophy gene defects are typically nonsense mutations or genomic rearrangements such as deletions, duplications or micro-deletions or insertions that disrupt the reading frame. As the human dystrophin gene is a large and complex gene with the 79 exons being spliced together to generate a mature mRNA with an open reading frame of approximately 11,000 bases, there are many positions where these mutations can occur. Consequently, a comprehensive antisense oligonucleotide based therapy to address many of the different disease-causing mutations in the dystrophin gene will require that many exons can be targeted for removal during the splicing process.

Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (i.e. splice donor sites, splice acceptor sites or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridisable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense molecule need not be 100% complementary to that of its target sequence to be specifically hybridisable. An antisense molecule is specifically hybridisable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

While the above method may be used to select antisense molecules capable of deleting any exon from within a protein that is capable of being shortened without affecting its biological function, the exon deletion should not lead to a reading frame shift in the shortened transcribed mRNA. Thus, if in a linear sequence of three exons the end of the first exon encodes two of three nucleotides in a codon and the next exon is deleted then the third exon in the linear sequence must start with a single nucleotide that is capable of completing the nucleotide triplet for a codon. If the third exon does not commence with a single nucleotide there will be a reading frame shift that would lead to the generation of truncated or a non-functional protein.

It will be appreciated that the codon arrangements at the end of exons in structural proteins may not always break at the end of a codon, consequently there may be a need to delete more than one exon from the pre-mRNA to ensure in-frame reading of the mRNA. In such circumstances, a plurality of antisense oligonucleotides may need to be selected by the method of the invention wherein each is directed to a different region responsible for inducing splicing in the exons that are to be deleted.

The length of an antisense molecule may vary so long as it is capable of binding selectively to the intended location within the pre-mRNA molecule. The length of such sequences can be determined in accordance with selection procedures described herein. Generally, the antisense molecule will be from about 10 nucleotides in length up to about 50 nucleotides in length. It will be appreciated however that any length of nucleotides within this range may be used in the method. Preferably, the length of the antisense molecule is between 17 to 30 nucleotides in length.

In order to determine which exons can be connected in a dystrophin gene, reference should be made to an exon boundary map. Connection of one exon with another is based on the exons possessing the same number at the 3' border as is present at the 5' border of the exon to which it is being connected. Therefore, if exon 7 were deleted, exon 6 must connect to either exons 12 or 18 to maintain the reading frame. Thus, antisense oligonucleotides would need to

be selected which redirected splicing for exons 7 to 11 in the first instance or exons 7 to 17 in the second instance. Another and somewhat simpler approach to restore the reading frame around an exon 7 deletion would be to remove the two flanking exons. Induction of exons 6 and 8 skipping should result in an in-frame transcript with the splicing of exons 5 to 9. In practise however, targeting exon 8 for removal from the pre-mRNA results in the co-removal of exon 9 so the resultant transcript would have exon 5 joined to exon 10. The inclusion or exclusion of exon 9 does not alter the reading frame. Once the antisense molecules to be tested have been identified, they are prepared according to standard techniques known in the art. The most common method for producing antisense molecules is the methylation of the 2' hydroxyribose position and the incorporation of a phosphorothioate backbone produces molecules that superficially resemble RNA but that are much more resistant to nuclease degradation.

To avoid degradation of pre-mRNA during duplex formation with the antisense molecules, the antisense molecules used in the method may be adapted to minimise or prevent cleavage by endogenous RNase H. This property is highly preferred as the treatment of the RNA with the unmethylated oligonucleotides either intracellularly or in crude extracts that contain RNase H leads to degradation of the pre-mRNA: antisense oligonucleotide duplexes. Any form of modified antisense molecules that is capable of by-passing or not inducing such degradation may be used in the present method. An example of antisense molecules which when duplexed with RNA are not cleaved by cellular RNase H is 2'-O-methyl derivatives. 2'-O-methyl-oligoribonucleotides are very stable in a cellular environment and in animal tissues, and their duplexes with RNA have higher Tm values than their ribo- or deoxyribo- counterparts.

Antisense molecules that do not activate RNase H can be made in accordance with known techniques (see, e.g., U.S. Pat. 5,149,797). Such antisense molecules, which may be deoxyribonucleotide or ribonucleotide sequences, simply contain any structural modification which sterically hinders or prevents binding of RNase H to a duplex molecule containing the oligonucleotide as one member thereof, which structural modification does not substantially hinder or

disrupt duplex formation. Because the portions of the oligonucleotide involved in duplex formation are substantially different from those portions involved in RNase H binding thereto, numerous antisense molecules that do not activate RNase H are available. For example, such antisense molecules may be oligonucleotides wherein at least one, or all, of the inter-nucleotide bridging phosphate residues are modified phosphates, such as methyl phosphonates, methyl phosphorothioates, phosphoromorpholidates, phosphoropiperazidates and phosphoramidates. For example, every other one of the internucleotide bridging phosphate residues may be modified as described. In another non-limiting example, such antisense molecules are molecules wherein at least one, or all, of the nucleotides contain a 2' lower alkyl moiety (e.g., C_1-C_4, linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl, and isopropyl). For example, every other one of the nucleotides may be modified as described.

While antisense oligonucleotides are a preferred form of the antisense molecules, the present invention comprehends other oligomeric antisense molecules, including but not limited to oligonucleotide mimetics such as are described below.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural inter-nucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their inter-nucleoside backbone can also be considered to be oligonucleosides.

In other preferred oligonucleotide mimetics, both the sugar and the inter-nucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization

properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleo-bases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. Certain nucleo-bases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds that are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense molecules, particularly oligonucleotides,

which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the increased resistance to nuclease degradation, increased cellular uptake, and an additional region for increased binding affinity for the target nucleic acid.

Methods of Manufacturing Antisense Molecules

The antisense molecules used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). One method for synthesising oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066.

Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives. In one such automated embodiment, diethyl-phosphoramidites are used as starting materials and may be synthesized as described by Beaucage, *et al.*, (1981) *Tetrahedron Letters*, **22**:1859-1862.

The antisense molecules of the invention are synthesised *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules. The molecules of the invention may also be mixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption.

Therapeutic Agents

The present invention also can be used as a prophylactic or therapeutic, which may be utilised for the purpose of treatment of a genetic disease.

Accordingly, in one embodiment the present invention provides antisense molecules that bind to a selected target in the dystrophin pre-mRNA to induce efficient and consistent exon skipping described herein in a therapeutically effective amount admixed with a pharmaceutically acceptable carrier, diluent, or excipient.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset and the like, when administered to a patient. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Martin, *Remington's Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

In a more specific form of the invention there are provided pharmaceutical compositions comprising therapeutically effective amounts of an antisense molecule together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength and additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol). The material may be incorporated into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g., Martin, Remington's Pharmaceutical Sciences*, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 that are herein incorporated by reference. The

compositions may be prepared in liquid form, or may be in dried powder, such as lyophilised form.

It will be appreciated that pharmaceutical compositions provided according to the present invention may be administered by any means known in the art. Preferably, the pharmaceutical compositions for administration are administered by injection, orally, or by the pulmonary, or nasal route. The antisense molecules are more preferably delivered by intravenous, intra-arterial, intraperitoneal, intramuscular, or subcutaneous routes of administration.

Antisense molecule based therapy

Also addressed by the present invention is the use of antisense molecules of the present invention, for manufacture of a medicament for modulation of a genetic disease.

The delivery of a therapeutically useful amount of antisense molecules may be achieved by methods previously published. For example, intracellular delivery of the antisense molecule may be via a composition comprising an admixture of the antisense molecule and an effective amount of a block copolymer. An example of this method is described in US patent application US 20040248833.

Other methods of delivery of antisense molecules to the nucleus are described in Mann CJ *et al.*, (2001) ["*Antisense-induced exon skipping and the synthesis of dystrophin in the mdx mouse*". Proc., Natl. Acad. Science, 98(1) 42-47] and in Gebski *et al.*, (2003). Human Molecular Genetics, 12(15): 1801-1811.

A method for introducing a nucleic acid molecule into a cell by way of an expression vector either as naked DNA or complexed to lipid carriers, is described in US patent US 6,806,084.

It may be desirable to deliver the antisense molecule in a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes or liposome formulations.

Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. These formulations may have net cationic, anionic or neutral charge characteristics and are useful characteristics with *in vitro*, *in vivo* and *ex vivo* delivery methods. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 .PHI.m can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, and DNA can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., Trends Biochem. Sci., 6:77, 1981).

In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the antisense molecule of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, et al., Biotechniques, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Alternatively, the antisense construct may be combined with other pharmaceutically acceptable carriers or diluents to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

The routes of administration described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and any dosage for any particular animal and condition.Multiple approaches for introducing functional new genetic material into cells, both in vitro and in vivo have been attempted (Friedmann (1989) Science, 244:1275-1280).

These approaches include integration of the gene to be expressed into modified retroviruses (Friedmann (1989) supra; Rosenberg (1991) Cancer Research 51(18), suppl.: 5074S-5079S); integration into non-retrovirus vectors (Rosenfeld, et al. (1992) Cell, 68:143-155; Rosenfeld, et al. (1991) Science, 252:431-434); or delivery of a transgene linked to a heterologous promoter-enhancer element via liposomes (Friedmann (1989), supra; Brigham, et al. (1989) Am. J. Med. Sci., 298:278-281; Nabel, et al. (1990) Science, 249:1285-1288; Hazinski, et al. (1991) Am. J. Resp. Cell Molec. Biol., 4:206-209; and Wang and Huang (1987) Proc. Natl. Acad. Sci. (USA), 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu (1988) J. Biol. Chem., 263:14621-14624) or the use of naked DNA, expression vectors (Nabel et al. (1990), supra); Wolff et al. (1990) Science, 247:1465-1468). Direct injection of transgenes into tissue produces only localized expression (Rosenfeld (1992) supra); Rosenfeld et al. (1991) supra; Brigham et al. (1989) supra; Nabel (1990) supra; and Hazinski et al. (1991) supra). The Brigham et al. group (Am. J. Med. Sci. (1989) 298:278-281 and Clinical Research (1991) 39 (abstract)) have reported in vivo transfection only of lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. An example of a review article of human gene therapy procedures is: Anderson, Science (1992) 256:808-813.

The antisense molecules of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such pro-drugs, and other bioequivalents.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, (including by nebulizer, intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intra-arterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Kits of the Invention

The invention also provides kits for treatment of a patient with a genetic disease which kit comprises at least an antisense molecule, packaged in a suitable container, together with instructions for its use.

In a preferred embodiment, the kits will contain at least one antisense molecule as shown in Table 1A, or a cocktail of antisense molecules as shown in Table 1B or a "weasel" compound as shown in Table 1C. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

Those of ordinary skill in the field should appreciate that applications of the above method has wide application for identifying antisense molecules suitable for use in the treatment of many other diseases.

EXAMPLES

The following Examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these Examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. The references cited herein are expressly incorporated by reference.

Methods of molecular cloning, immunology and protein chemistry, which are not explicitly described in the following examples, are reported in the literature and are known by those skilled in the art. General texts that described conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art, included, for example: Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); Glover ed., *DNA Cloning: A Practical Approach*, Volumes I and II, MRL Press, Ltd., Oxford, U.K. (1985); and Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. *Current Protocols in Molecular Biology.* Greene Publishing Associates/Wiley Intersciences, New York (2002).

Determining Induced Exon Skipping in Human Muscle Cells

Attempts by the inventors to develop a rational approach in antisense molecules design were not completely successful as there did not appear to be a consistent trend that could be applied to all exons. As such, the identification of the most effective and therefore most therapeutic antisense molecules compounds has been the result of empirical studies.

These empirical studies involved the use of computer programs to identify motifs potentially involved in the splicing process. Other computer programs were also used to identify regions of the pre-mRNA which may not have had extensive secondary structure and therefore potential sites for annealing of antisense molecules. Neither of these approaches proved completely reliable in designing antisense oligonucleotides for reliable and efficient induction of exon skipping.

Annealing sites on the human dystrophin pre-mRNA were selected for examination, initially based upon known or predicted motifs or regions involved in splicing. 2OMe antisense oligonucleotides were designed to be complementary to the target sequences under investigation and were synthesised on an Expedite 8909 Nucleic Acid Synthesiser. Upon completion of synthesis, the oligonucleotides were cleaved from the support column and de-protected in ammonium hydroxide before being desalted. The quality of the oligonucleotide synthesis was monitored by the intensity of the trityl signals upon each deprotection step during the synthesis as detected in the synthesis log. The concentration of the antisense oligonucleotide was estimated by measuring the absorbance of a diluted aliquot at 260nm.

Specified amounts of the antisense molecules were then tested for their ability to induce exon skipping in an *in vitro* assay, as described below.

Briefly, normal primary myoblast cultures were prepared from human muscle biopsies obtained after informed consent. The cells were propagated and allowed to differentiate into myotubes using standard culturing techniques. The cells were then transfected with the antisense oligonucleotides by delivery of the

oligonucleotides to the cells as cationic lipoplexes, mixtures of antisense molecules or cationic liposome preparations.

The cells were then allowed to grow for another 24 hours, after which total RNA was extracted and molecular analysis commenced. Reverse transcriptase amplification (RT-PCR) was undertaken to study the targeted regions of the dystrophin pre-mRNA or induced exonic re-arrangements.

For example, in the testing of an antisense molecule for inducing exon 19 skipping the RT-PCR test scanned several exons to detect involvement of any adjacent exons. For example, when inducing skipping of exon 19, RT-PCR was carried out with primers that amplified across exons 17 and 21. Amplifications of even larger products in this area (i.e. exons 13-26) were also carried out to ensure that there was minimal amplification bias for the shorter induced skipped transcript. Shorter or exon skipped products tend to be amplified more efficiently and may bias the estimated of the normal and induced transcript.

The sizes of the amplification reaction products were estimated on an agarose gel and compared against appropriate size standards. The final confirmation of identity of these products was carried out by direct DNA sequencing to establish that the correct or expected exon junctions have been maintained.

Once efficient exon skipping had been induced with one antisense molecule, subsequent overlapping antisense molecules may be synthesized and then evaluated in the assay as described above. Our definition of an efficient antisense molecule is one that induces strong and sustained exon skipping at transfection concentrations in the order of 300 nM or less.

<u>**Antisense Oligonucleotides Directed at Exon 8**</u>

Antisense oligonucleotides directed at exon 8 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 3 shows differing efficiencies of two antisense molecules directed at exon 8 acceptor splice site. H8A(-06+18) [SEQ ID NO:1], which anneals to the last 6

bases of intron 7 and the first 18 bases of exon 8, induces substantial exon 8 and 9 skipping when delivered into cells at a concentration of 20 nM. The shorter antisense molecule, H8A(-06+14) [SEQ ID NO: 4] was only able to induce exon 8 and 9 skipping at 300 nM, a concentration some 15 fold higher than H8A(-06+18), which is the preferred antisense molecule.

This data shows that some particular antisense molecules induce efficient exon skipping while another antisense molecule, which targets a near-by or overlapping region, can be much less efficient. Titration studies show one compound is able to induce targeted exon skipping at 20 nM while the less efficient antisense molecules only induced exon skipping at concentrations of 300 nM and above. Therefore, we have shown that targeting of the antisense molecules to motifs involved in the splicing process plays a crucial role in the overall efficacy of that compound.

Efficacy refers to the ability to induce consistent skipping of a target exon. However, sometimes skipping of the target exons is consistently associated with a flanking exon. That is, we have found that the splicing of some exons is tightly linked. For example, in targeting exon 23 in the mouse model of muscular dystrophy with antisense molecules directed at the donor site of that exon, dystrophin transcripts missing exons 22 and 23 are frequently detected. As another example, when using an antisense molecule directed to exon 8 of the human dystrophin gene, all induced transcripts are missing both exons 8 and 9. Dystrophin transcripts missing only exon 8 are not observed.

Table 2 below discloses antisense molecule sequences that induce exon 8 (and 9) skipping.

Antisense Oligonucleotide name	**Sequence**	**Ability to induce skipping**
H8A(-06+18)	5'-GAU AGG UGG UAU CAA CAU CUG UAA	Very strong to 20 nM
H8A (-03+18)	5' – GAU AGG UGG UAU CAA CAU CUG	Very strong skipping to 40nM
H8A(-07+18)	5' – GAU AGG UGG UAU CAA CAU CUG UAA G	Strong skipping to 40nM

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H8A(-06+14)	5' – GGU GGU AUC AAC AUC UGU AA	Skipping to 300nM
H8A(-10+10)	5' – GUA UCA ACA UCU GUA AGC AC	Patchy/weak skipping to 100nm

Table 2

Antisense Oligonucleotides Directed at Exon 7

Antisense oligonucleotides directed at exon 7 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 4 shows the preferred antisense molecule, H7A(+45+67) [SEQ ID NO: 6], and another antisense molecule, H7A(+2+26) [SEQ ID NO: 7], inducing exon 7 skipping. Nested amplification products span exons 3 to 9. Additional products above the induced transcript missing exon 7 arise from amplification from carry-over outer primers from the RT-PCR as well as heteroduplex formation.

Table 3 below discloses antisense molecule sequences for induced exon 7 skipping.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H7A(+45+67)	5' – UGC AUG UUC CAG UCG UUG UGU GG	Strong skipping to 20nM
H7A(+02+26)	5' – CAC UAU UCC AGU CAA AUA GGU CUG G	Weak skipping at 100nM
H7D(+15-10)	5' – AUU UAC CAA CCU UCA GGA UCG AGU A	Weak skipping to 300nM
H7A(-18+03)	5' - GGC CUA AAA CAC AUA CAC AUA	Weak skipping to 300nM

Table 3

Antisense Oligonucleotides Directed at Exon 6

Antisense oligonucleotides directed at exon 6 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 5 shows an example of two non-preferred antisense molecules inducing very low levels of exon 6 skipping in cultured human cells. Targeting this exon for specific removal was first undertaken during a study of the canine model using the oligonucleotides as listed in Table 4, below. Some of the human specific oligonucleotides were also evaluated, as shown in Figure 5. In this example, both antisense molecules target the donor splice site and only induced low levels of exon 6 skipping. Both H6D(+4-21) [SEQ ID NO: 17] and H6D(+18-4) [SEQ ID NO: 18] would be regarded as non-preferred antisense molecules.

One antisense oligonucleotide that induced very efficient exon 6 skipping in the canine model, C6A(+69+91) [SEQ ID NO: 14], would anneal perfectly to the corresponding region in human dystrophin exon 6. This compound was evaluated, found to be highly efficient at inducing skipping of that target exon, as shown in Figure 6 and is regarded as the preferred compound for induced exon 6 skipping. Table 4 below discloses antisense molecule sequences for induced exon 6 skipping.

Antisense Oligo name	Sequence	Ability to induce skipping
C6A(-10+10)	5' CAU UUU UGA CCU ACA UGU GG	No skipping
C6A(-14+06)	5' UUU GAC CUA CAU GUG GAA AG	No skipping
C6A(-14+12)	5' UAC AUU UUU GAC CUA CAU GUG GAA AG	No skipping
C6A(-13+09)	5' AUU UUU GAC CUA CAU GGG AAA G	No skipping
CH6A(+69+91)	5' UAC GAG UUG AUU GUC GGA CCC AG	Strong skipping to 20 nM
C6D(+12-13)	5' GUG GUC UCC UUA CCU AUG ACU GUG G	Weak skipping at 300 nM
C6D(+06-11)	5' GGU CUC CUU ACC UAU GA	No skipping
H6D(+04-21)	5' UGU CUC AGU AAU CUU CUU ACC UAU	Weak skipping to 50 nM
H6D(+18-04)	5' UCU UAC CUA UGA CUA UGG AUG AGA	Very weak skipping to 300 nM

Table 4

Antisense Oligonucleotides Directed at Exon 4

Antisense oligonucleotides directed at exon 4 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 7 shows an example of a preferred antisense molecule inducing skipping of exon 4 skipping in cultured human cells. In this example, one preferred antisense compound, H4A(+13+32) [SEQ ID NO:19], which targeted a presumed exonic splicing enhancer induced efficient exon skipping at a concentration of 20 nM while other non-preferred antisense oligonucleotides failed to induce even low levels of exon 4 skipping. Another preferred antisense molecule inducing skipping of exon 4 was H4A(+111+40) [SEQ ID NO:22], which induced efficient exon skipping at a concentration of 20 nM.

Table 5 below discloses antisense molecule sequences for inducing exon 4 skipping.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H4A(+13+32)	5' GCA UGA ACU CUU GUG GAU CC	Skipping to 20 nM
H4A(+11+40)	5'UGU UCA GGG CAU GAA CUC UUG UGG AUC CUU	Skipping to 20 nM
H4D(+04-16)	5' CCA GGG UAC UAC UUA CAU UA	No skipping
H4D(-24-44)	5' AUC GUG UGU CAC AGC AUC CAG	No skipping

Table 5

Antisense Oligonucleotides Directed at Exon 3

Antisense oligonucleotides directed at exon 3 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H3A(+30+60) [SEQ ID NO:23] induced substantial exon 3 skipping when delivered into cells at a concentration of 20 nM to 600 nM. The antisense molecule, H3A(+35+65) [SEQ ID NO: 24] induced exon skipping at 300 nM.

Table 6 below discloses antisense molecule sequences that induce exon 3 skipping.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H3A(+30+60)	UAG GAG GCG CCU CCC AUC CUG UAG GUC ACU G	Moderate skipping to 20 to 600 nM
H3A(+35+65)	AGG UCU AGG AGG CGC CUC CCA UCC UGU AGG U	Working to 300 nM
H3A(+30+54)	GCG CCU CCC AUC CUG UAG GUC ACU G	Moderate 100-600 nM
H3D(+46-21)	CUU CGA GGA GGU CUA GGA GGC GCC UC	No skipping
H3A(+30+50)	CUC CCA UCC UGU AGG UCA CUG	Moderate 20-600 nM
H3D(+19-03)	UAC CAG UUU UUG CCC UGU CAG G	No skipping
H3A(-06+20)	UCA AUA UGC UGC UUCCCA AAC UGA AA	No skipping
H3A(+37+61)	CUA GGA GGC GCC UCC CAU CCU GUA G	No skipping

Table 6

Antisense Oligonucleotides Directed at Exon 5

Antisense oligonucleotides directed at exon 5 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H5A(+20+50) [SEQ ID NO:31] induces substantial exon 5 skipping when delivered into cells at a concentration of 100 nM. Table 7 below shows other antisense molecules tested. The majority of these antisense molecules were not as effective at exon skipping as H5A(+20+50). However, H5A(+15+45) [SEQ ID NO: 40] was able to induce exon 5 skipping at 300 nM.

Table 7 below discloses antisense molecule sequences that induce exon 5 skipping.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H5A(+20+50)	UUA UGA UUU CCA UCU ACG AUG UCA GUA CUU C	Working to 100 nM
H5D(+25-05)	CUU ACC UGC CAG UGG AGG AUU AUA UUC CAA A	No skipping
H5D(+10-15)	CAU CAG GAU UCU UAC CUG CCA GUG G	Inconsistent at 300 nM

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H5A(+10+34)	CGA UGU CAG UAC UUC CAA UAU UCA C	Very weak
H5D(-04-21)	ACC AUU CAU CAG GAU UCU	No skipping
H5D(+16-02)	ACC UGC CAG UGG AGG AUU	No skipping
H5A(-07+20)	CCA AUA UUC ACU AAA UCA ACC UGU UAA	No skipping
H5D(+18-12)	CAG GAU UCU UAC CUG CCA GUG GAG GAU UAU	No skipping
H5A(+05+35)	ACG AUG UCA GUA CUU CCA AUA UUC ACU AAA U	No skipping
H5A(+15+45)	AUU UCC AUC UAC GAU GUC AGU ACU UCC AAU A	Working to 300 nM

Table 7

Antisense Oligonucleotides Directed at Exon 10

Antisense oligonucleotides directed at exon 10 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H10A(-05+16) [SEQ ID NO:41] induced substantial exon 10 skipping when delivered into cells. Table 8 below shows other antisense molecules tested. The antisense molecules ability to induce exon skipping was variable. Table 8 below discloses antisense molecule sequences that induce exon 10 skipping.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H10A(-05+16)	CAG GAG CUU CCA AAU GCU GCA	Not tested
H10A(-05+24)	CUU GUC UUC AGG AGC UUC CAA AUG CUG CA	Not tested
H10A(+98+119)	UCC UCA GCA GAA AGA AGC CAC G	Not tested
H10A(+130+149)	UUA GAA AUC UCU CCU UGU GC	No skipping
H10A(-33-14)	UAA AUU GGG UGU UAC ACA AU	No skipping

Table 8

Antisense Oligonucleotides Directed at Exon 11

Antisense oligonucleotides directed at exon 11 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 8B shows an example of H11A(+75+97) [SEQ ID NO:49] antisense molecule inducing exon 11 skipping in cultured human cells. H11A(+75+97) induced substantial exon 11 skipping when delivered into cells at a concentration of 5 nM. Table 9 below shows other antisense molecules tested. The antisense molecules ability to induce exon skipping was observed at 100 nM.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H11D(+26+49)	CCC UGA GGC AUU CCC AUC UUG AAU	Skipping at 100 nM
H11D(+11-09)	AGG ACU UAC UUG CUU UGU UU	Skipping at 100 nM
H11A(+118+140)	CUU GAA UUU AGG AGA UUC AUC UG	Skipping at 100 nM
H11A(+75+97)	CAU CUU CUG AUA AUU UUC CUG UU	Skipping at 100 nM
H11D(+26+49)	CCC UGA GGC AUU CCC AUC UUG AAU	Skipping at 5nM

Table 9

Antisense Oligonucleotides Directed at Exon 12

Antisense oligonucleotides directed at exon 12 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H12A(+52+75) [SEQ ID NO:50] induced substantial exon 12 skipping when delivered into cells at a concentration of 5 nM, as shown in Figure 8A. Table 10 below shows other antisense molecules tested at a concentration range of 5, 25, 50, 100, 200 and 300 nM. The antisense molecules ability to induce exon skipping was variable.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H12A(+52+75)	UCU UCU GUU UUU GUU AGC CAG UCA	Skipping at 5 nM
H12A(-10+10)	UCU AUG UAA ACU GAA AAU UU	Skipping at 100 nM
H12A(+11+30)	UUC UGG AGA UCC AUU AAA AC	No skipping

Table 10

Antisense Oligonucleotides Directed at Exon 13

Antisense oligonucleotides directed at exon 13 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H13A(+77+100) [SEQ ID NO:53] induced substantial exon 13 skipping when delivered into cells at a concentration of 5 nM. Table 11 below includes two other antisense molecules tested at a concentration range of 5, 25, 50, 100, 200 and 300 nM. These other antisense molecules were unable to induce exon skipping.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H13A(+77+100)	CAG CAG UUG CGU GAU CUC CAC UAG	Skipping at 5 nM
H13A(+55+75)	UUC AUC AAC UAC CAC CAC CAU	No skipping
H13D(+06-19)	CUA AGC AAA AUA AUC UGA CCU UAA G	No skipping

Table 11

Antisense Oligonucleotides Directed at Exon 14

Antisense oligonucleotides directed at exon 14 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H14A(+37+64) [SEQ ID NO:56] induced weak exon 14 skipping when delivered into cells at a concentration of 100 nM. Table 12 below includes other antisense molecules tested at a concentration range of 5, 25, 50, 100, 200 and 300 nM. The other antisense molecules were unable to induce exon skipping at any of the concentrations tested.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H14A(+37+64)	CUU GUA AAA GAA CCC AGC GGU CUU CUG U	Skipping at 100 nM
H14A(+14+35)	CAU CUA CAG AUG UUU GCC CAU C	No skipping
H14A(+51+73)	GAA GGA UGU CUU GUA AAA GAA CC	No skipping
H14D(-02+18)	ACC UGU UCU UCA GUA AGA CG	No skipping
H14D(+14-10)	CAU GAC ACA CCU GUU CUU CAG UAA	No skipping

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H14A(+61+80)	CAU UUG AGA AGG AUG UCU UG	No skipping
H14A(-12+12)	AUC UCC CAA UAC CUG GAG AAG AGA	No skipping

Table 12

Antisense Oligonucleotides Directed at Exon 15

Antisense oligonucleotides directed at exon 15 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H15A(-12+19) [SEQ ID NO:63] and H15A(+48+71) [SEQ ID NO:64] induced substantial exon 15 skipping when delivered into cells at a concentration of 10 Nm, as shown in Figure 9A. Table 13 below includes other antisense molecules tested at a concentration range of 5, 25, 50, 100, 200 and 300 Nm. These other antisense molecules were unable to induce exon skipping at any of the concentrations tested.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H15A(-12+19)	GCC AUG CAC UAA AAA GGC ACU GCA AGA CAU U	Skipping at 5 Nm
H15A(+48+71)	UCU UUA AAG CCA GUU GUG UGA AUC	Skipping at 5 Nm
H15A(+08+28)	UUU CUG AAA GCC AUG CAC UAA	No skipping
H15A(-12+19)	GCC AUG CAC UAA AAA GGC ACU GCA AGA CAU U	No skipping
H15D(+17-08)	GUA CAU ACG GCC AGU UUU UGA AGA C	No skipping

Table 13

Antisense Oligonucleotides Directed at Exon 16

Antisense oligonucleotides directed at exon 16 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H16A(-12+19) [SEQ ID NO:67] and H16A(-06+25) [SEQ ID NO:68] induced substantial exon 16 skipping when delivered into cells at a concentration of 10 nM, as shown in Figure 9B. Table 14 below includes other antisense molecules

tested. H16A(-06+19) [SEQ ID NO:69] and H16A(+87+109) [SEQ ID NO:70] were tested at a concentration range of 5, 25, 50, 100, 200 and 300 nM. These two antisense molecules were able to induce exon skipping at 25 nM and 100 nM, respectively. Additional antisense molecules were tested at 100, 200 and 300 nM and did not result in any exon skipping.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H16A(-12+19)	CUA GAU CCG CUU UUA AAA CCU GUU AAA ACA A	Skipping at 5 nM
H16A(-06+25)	UCU UUU CUA GAU CCG CUU UUA AAA CCU GUU A	Skipping at 5 nM
H16A(-06+19)	CUA GAU CCG CUU UUA AAA CCU GUU A	Skipping at 25 nM
H16A(+87+109)	CCG UCU UCU GGG UCA CUG ACU UA	Skipping at 100 nM
H16A(-07+19)	CUA GAU CCG CUU UUA AAA CCU GUU AA	No skipping
H16A(-07+13)	CCG CUU UUA AAA CCU GUU AA	No skipping
H16A(+12+37)	UGG AUU GCU UUU UCU UUU CUA GAU CC	No skipping
H16A(+92+116)	CAU GCU UCC GUC UUC UGG GUC ACU G	No skipping
H16A(+45+67)	G AUC UUG UUU GAG UGA AUA CAG U	No skipping
H16A(+105+126)	GUU AUC CAG CCA UGC UUC CGU C	No skipping
H16D(+05-20)	UGA UAA UUG GUA UCA CUA ACC UGU G	No skipping
H16D(+12-11)	GUA UCA CUA ACC UGU GCU GUA C	No skipping

Table 14

<u>Antisense Oligonucleotides Directed at Exon 19</u>

Antisense oligonucleotides directed at exon 19 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H19A(+35+65) [SEQ ID NO:79] induced substantial exon 19 skipping when delivered into cells at a concentration of 10 nM. This antisense molecule also showed very strong exon skipping at concentrations of 25, 50, 100, 300 and 600 nM.

Figure 10 illustrates exon 19 and 20 skipping using a “cocktail” of antisense oligonucleotides, as tested using gel electrophoresis. It is interesting to note that it was not easy to induce exon 20 skipping using single antisense oligonucleotides H20A(+44+71) [SEQ ID NO:81] or H20A(+149+170) [SEQ ID

NO:82], as illustrated in sections 2 and 3 of the gel shown in Figure 10. Whereas, a "cocktail" of antisense oligonucleotides was more efficient as can be seen in section 4 of Figure 10 using a "cocktail" of antisense oligonucleotides H20A(+44+71) and H20A(+149+170). When the cocktail was used to target exon 19, skipping was even stronger (see section 5, Figure 10).

Figure 11 illustrates gel electrophoresis results of exon 19/20 skipping using "weasels" The "weasels" were effective in skipping exons 19 and 20 at concentrations of 25, 50, 100, 300 and 600 nM. A further "weasel" sequence is shown in the last row of Table 3C. This compound should give good results.

Antisense Oligonucleotides Directed at Exon 20

Antisense oligonucleotides directed at exon 20 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

None of the antisense oligonucleotides tested induced exon 20 skipping when delivered into cells at a concentration of 10, 25, 50, 300 or 600 nM (see Table 15). Antisense molecules H20A(-11+17) [SEQ ID NO:86] and H20D(+08-20) [SEQ ID NO:87] are yet to be tested.

However, a combination or "cocktail" of H20A(+44+71) [SEQ ID NO: 81] and H20(+149+170) [SEQ ID NO:82] in a ratio of 1:1, exhibited very strong exon skipping at a concentration of 100 nM and 600 nM. Further, a combination of antisense molecules H19A(+35+65) [SEQ ID NO:79], H20A(+44+71) [SEQ ID NO:81] and H20A(+149+170) [SEQ ID NO:82] in a ratio of 2:1:1, induced very strong exon skipping at a concentration ranging from 10 nM to 600 nM.

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H20A(+44+71)	CUG GCA GAA UUC GAU CCA CCG GCU GUU C	No skipping
H20A(+149+170)	CAG CAG UAG UUG UCA UCU GCU C	No skipping
H20A(+185+203)	UGA UGG GGU GGU GGG UUG G	No skipping
H20A(-08+17)	AUC UGC AUU AAC ACC CUC UAG AAA G	No skipping
H20A(+30+53)	CCG GCU GUU CAG UUG UUC UGA GGC	No skipping
H20A(-11+17)	AUC UGC AUU AAC ACC CUC UAG AAA GAA A	Not tested yet

Antisense Oligonucloetide name	Sequence	Ability to induce skipping
H20D(+08-20)	GAA GGA GAA GAG AUU CUU ACC UUA CAA A	Not tested yet
H20A(+44+71) & H20A(+149+170)	CUG GCA GAA UUC GAU CCA CCG GCU GUU C CAG CAG UAG UUG UCA UCU GCU C	Very strong skipping
H19A(+44+71) : H20A(+44+71); H20A(+149+170)	GCC UGA GCU GAU CUG CUG GCA UCU UGC AGU U CUG GCA GAA UUC GAU CCA CCG GCU GUU C CAG CAG UAG UUG UCA UCU GCU C	Very strong skipping

Table 15

Antisense Oligonucleotides Directed at Exon 21

Antisense oligonucleotides directed at exon 21 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H21A(+85+108) [SEQ ID NO:92] and H21A(+85+106) [SEQ ID NO:91] induced exon 21 skipping when delivered into cells at a concentration of 50 nM. Table 16 below includes other antisense molecules tested at a concentration range of 5, 25, 50, 100, 200 and 300 nM. These antisense molecules showed a variable ability to induce exon skipping

Antisense Oligonucleotide name	Sequence	Ability to induce skipping
H21A(-06+16)	GCC GGU UGA CUU CAU CCU GUG C	Skips at 600 nM
H21A(+85+106)	CUG CAU CCA GGA ACA UGG GUC C	Skips at 50 nM
H21A(+85+108)	GUC UGC AUC CAG GAA CAU GGG UC	Skips at 50 nM
H21A(+08+31)	GUU GAA GAU CUG AUA GCC GGU UGA	Skips faintly to
H21D(+18-07)	UAC UUA CUG UCU GUA GCU CUU UCU	No skipping

Table 16

Antisense Oligonucleotides Directed at Exon 22

Antisense oligonucleotides directed at exon 22 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 12 illustrates differing efficiencies of two antisense molecules directed at exon 22 acceptor splice site. H22A(+125+106) [SEQ ID NO:96] and H22A(+80+101) [SEQ ID NO: 98] induce strong exon 22 skipping from 50 nM to 600 nM concentration.

H22A(+125+146) [SEQ ID NO:96] and H22A(+80+101) [SEQ ID NO:98] induced exon 22 skipping when delivered into cells at a concentration of 50 nM. Table 17 below shows other antisense molecules tested at a concentration range of 50, 100, 300 and 600 nM. These antisense molecules showed a variable ability to induce exon skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H22A(+22+45)	CAC UCA UGG UCU CCU GAU AGC GCA	No skipping
H22A(+125+146)	CUG CAA UUC CCC GAG UCU CUG C	Skipping to 50 nM
H22A(+47+69)	ACU GCU GGA CCC AUG UCC UGA UG	Skipping to 300 nM
H22A(+80+101)	CUA AGU UGA GGU AUG GAG AGU	Skipping to 50 nM
H22D(+13-11)	UAU UCA CAG ACC UGC AAU UCC CC	No skipping

Table 17

Antisense Oligonucleotides Directed at Exon 23

Antisense oligonucleotides directed at exon 23 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Table 18 below shows antisense molecules tested at a concentration range of 25, 50, 100, 300 and 600 nM. These antisense molecules showed no ability to induce exon skipping or are yet to be tested.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H23A(+34+59)	ACA GUG GUG CUG AGA UAG UAU AGG CC	No skipping
H23A(+18+39)	UAG GCC ACU UUG UUG CUC UUG C	No Skipping
H23A(+72+90)	UUC AGA GGG CGC UUU CUU C	No Skipping

Table 18

Antisense Oligonucleotides Directed at Exon 24

Antisense oligonucleotides directed at exon 24 were prepared using similar methods as described above. Table 19 below outlines the antisense oligonucleotides directed at exon 24 that are yet to be tested for their ability to induce exon 24 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H24A(+48+70)	GGG CAG GCC AUU CCU CCU UCA GA	Needs testing
H24A(-02+22)	UCU UCA GGG UUU GUA UGU GAU UCU	Needs testing

Table 19

Antisense Oligonucleotides Directed at Exon 25

Antisense oligonucleotides directed at exon 25 were prepared using similar methods as described above. Table 20 below shows the antisense oligonucleotides directed at exon 25 that are yet to be tested for their ability to induce exon 25 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H25A(+9+36)	CUG GGC UGA AUU GUC UGA AUA UCA CUG	Needs testing
H25A(+131+156)	CUG UUG GCA CAU GUG AUC CCA CUG AG	Needs testing
H25D(+16-08)	GUC UAU ACC UGU UGG CAC AUG UGA	Needs testing

Table 20

Antisense Oligonucleotides Directed at Exon 26

Antisense oligonucleotides directed at exon 26 were prepared using similar methods as described above. Table 21 below outlines the antisense oligonucleotides directed at exon 26 that are yet to be tested for their ability to induce exon 26 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H26A(+132+156)	UGC UUU CUG UAA UUC AUC UGG AGU U	Needs testing
H26A(-07+19)	CCU CCU UUC UGG CAU AGA CCU UCC AC	Needs testing
H26A(+68+92)	UGU GUC AUC CAU UCG UGC AUC UCU G	Faint skipping at 600 nM

Table 21

<u>Antisense Oligonucleotides Directed at Exon 27</u>

Antisense oligonucleotides directed at exon 27 were prepared using similar methods as described above. Table 22 below outlines the antisense oligonucleotides directed at exon 27 that are yet to be tested for their ability to induce exon 27 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H27A(+82+106)	UUA AGG CCU CUU GUG CUA CAG GUG G	Needs testing
H27A(-4+19)	GGG CCU CUU CUU UAG CUC UCU GA	Faint skipping at 600 and 300 nM
H27D(+19-03)	GAC UUC CAA AGU CUU GCA UUU C	v. strong skipping at 600 and 300 nM

Table 22

<u>Antisense Oligonucleotides Directed at Exon 28</u>

Antisense oligonucleotides directed at exon 28 were prepared using similar methods as described above. Table 23 below outlines the antisense oligonucleotides directed at exon 28 that are yet to be tested for their ability to induce exon 28 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H28A(-05+19)	GCC AAC AUG CCC AAA CUU CCU AAG	v. strong skipping at 600 and 300 nM
H28A(+99+124)	CAG AGA UUU CCU CAG CUC CGC CAG GA	Needs testing
H28D(+16-05)	CUU ACA UCU AGC ACC UCA GAG	v. strong skipping at 600 and 300 nM

Table 23

Antisense Oligonucleotides Directed at Exon 29

Antisense oligonucleotides directed at exon 29 were prepared using similar methods as described above. Table 24 below outlines the antisense oligonucleotides directed at exon 29 that are yet to be tested for their ability to induce exon 29 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H29A(+57+81)	UCC GCC AUC UGU UAG GGU CUG UGC C	Needs testing
H29A(+18+42)	AUU UGG GUU AUC CUC UGA AUG UCG C	v. strong skipping at 600 and 300 nM
H29D(+17-05)	CAU ACC UCU UCA UGU AGU UCC C	v. strong skipping at 600 and 300 nM

Table 24

Antisense Oligonucleotides Directed at Exon 30

Antisense oligonucleotides directed at exon 30 were prepared using similar methods as described above. Table 25 below outlines the antisense oligonucleotides directed at exon 30 that are yet to be tested for their ability to induce exon 30 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H30A(+122+147)	CAU UUG AGC UGC GUC CAC CUU GUC UG	Needs testing
H30A(+25+50)	UCC UGG GCA GAC UGG AUG CUC UGU UC	Very strong skipping at 600 and 300 nM.
H30D(+19-04)	UUG CCU GGG CUU CCU GAG GCA UU	Very strong skipping at 600 and 300 nM.

Table 25

Antisense Oligonucleotides Directed at Exon 31

Antisense oligonucleotides directed at exon 31 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 13 illustrates differing efficiencies of two antisense molecules directed at exon 31 acceptor splice site and a "cocktail" of exon 31 antisense oligonucleotides at varying concentrations. H31D(+03-22) [SEQ ID NO:124] substantially induced exon 31 skipping when delivered into cells at a concentration of 20 nM. Table 26 below also includes other antisense molecules tested at a concentration of 100 and 300 nM. These antisense molecules showed a variable ability to induce exon skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H31D(+06-18)	UUC UGA AAU AAC AUA UAC CUG UGC	Skipping to 300 nM
H31D(+03-22)	UAG UUU CUG AAA UAA CAU AUA CCU G	Skipping to 20 nM
H31A(+05+25)	GAC UUG UCA AAU CAG AUU GGA	No skipping
H31D(+04-20)	GUU UCU GAA AUA ACA UAU ACC UGU	Skipping to 300 nM

Table 26

<u>Antisense Oligonucleotides Directed at Exon 32</u>

Antisense oligonucleotides directed at exon 32 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H32D(+04-16) [SEQ ID NO:127] and H32A(+49+73) [SEQ ID NO:130] induced exon 32 skipping when delivered into cells at a concentration of 300 nM. Table 27 below also shows other antisense molecules tested at a concentration of 100 and 300 nM. These antisense molecules did not show an ability to induce exon skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H32D(+04-16)	CAC CAG AAA UAC AUA CCA CA	Skipping to 300 nM
H32A(+151+170)	CAA UGA UUU AGC UGU GAC UG	No skipping
H32A(+10+32)	CGA AAC UUC AUG GAG ACA UCU UG	No skipping
H32A(+49+73)	CUU GUA GAC GCU GCU CAA AAU UGG C	Skipping to 300 nM

Table 27

Antisense Oligonucleotides Directed at Exon 33

Antisense oligonucleotides directed at exon 33 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 14 shows differing efficiencies of two antisense molecules directed at exon 33 acceptor splice site. H33A(+64+88) [SEQ ID NO:134] substantially induced exon 33 skipping when delivered into cells at a concentration of 10 nM. Table 28 below includes other antisense molecules tested at a concentration of 100, 200 and 300 nM. These antisense molecules showed a variable ability to induce exon skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H33D(+09-11)	CAU GCA CAC ACC UUU GCU CC	No skipping
H33A(+53+76)	UCU GUA CAA UCU GAC GUC CAG UCU	Skipping to 200 nM
H33A(+30+56)	GUC UUU AUC ACC AUU UCC ACU UCA GAC	Skipping to 200 nM
H33A(+64+88)	CCG UCU GCU UUU UCU GUA CAA UCU G	Skipping to 10 nM

Table 28

Antisense Oligonucleotides Directed at Exon 34

Antisense oligonucleotides directed at exon 34 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Table 29 below includes antisense molecules tested at a concentration of 100 and 300 nM. These antisense molecules showed a variable ability to induce exon skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H34A(+83+104)	UCC AUA UCU GUA GCU GCC AGC C	No skipping
H34A(+143+165)	CCA GGC AAC UUC AGA AUC CAA AU	No skipping
H34A(-20+10)	UUU CUG UUA CCU GAA AAG AAU UAU AAU GAA	Not tested
H34A(+46+70)	CAU UCA UUU CCU UUC GCA UCU UAC G	Skipping to 300 nM

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H34A(+95+120)	UGA UCU CUU UGU CAA UUC CAU AUC UG	Skipping to 300 nM
H34D(+10-20)	UUC AGU GAU AUA GGU UUU ACC UUU CCC CAG	Not tested
H34A(+72+96)	CUG UAG CUG CCA GCC AUU CUG UCA AG	No skipping

Table 29

Antisense Oligonucleotides Directed at Exon 35

Antisense oligonucleotides directed at exon 35 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 15 shows differing efficiencies of antisense molecules directed at exon 35 acceptor splice site. H35A(+24+43) [SEQ ID NO:144] substantially induced exon 35 skipping when delivered into cells at a concentration of 20 nM. Table 30 below also includes other antisense molecules tested at a concentration of 100 and 300 nM. These antisense molecules showed no ability to induce exon skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H35A(+141+161)	UCU UCU GCU CGG GAG GUG ACA	Skipping to 20 nM
H35A(+116+135)	CCA GUU ACU AUU CAG AAG AC	No skipping
H35A(+24+43)	UCU UCA GGU GCA CCU UCU GU	No skipping

Table 30

Antisense Oligonucleotides Directed at Exon 36

Antisense oligonucleotides directed at exon 36 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Antisense molecule H36A(+26+50) [SEQ ID NO:145] induced exon 36 skipping when delivered into cells at a concentration of 300 nM, as shown in Figure 16.

Antisense Oligonucleotides Directed at Exon 37

Antisense oligonucleotides directed at exon 37 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 17 shows differing efficiencies of two antisense molecules directed at exon 37 acceptor splice site. H37A(+82+105) [SEQ ID NO:148] and H37A(+134+157) [SEQ ID NO:149] substantially induced exon 37 skipping when delivered into cells at a concentration of 10 nM. Table 31 below shows the antisense molecules tested.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H37A(+26+50)	CGU GUA GAG UCC ACC UUU GGG CGU A	No skipping
H37A(+82+105)	UAC UAA UUU CCU GCA GUG GUC ACC	Skipping to 10 nM
H37A(+134+157)	UUC UGU GUG AAA UGG CUG CAA AUC	Skipping to 10 nM

Table 31

Antisense Oligonucleotides Directed at Exon 38

Antisense oligonucleotides directed at exon 38 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 18 illustrates antisense molecule H38A(+88+112) [SEQ ID NO:152] , directed at exon 38 acceptor splice site. H38A(+88+112) substantially induced exon 38 skipping when delivered into cells at a concentration of 10 nM. Table 32 below shows the antisense molecules tested and their ability to induce exon skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H38A(-01+19)	CCU UCA AAG GAA UGG AGG CC	No skipping
H38A(+59+83)	UGC UGA AUU UCA GCC UCC AGU GGU U	Skipping to 10 nM
H38A(+88+112)	UGA AGU CUU CCU CUU UCA GAU UCA C	Skipping to 10 nM

Table 32

Antisense Oligonucleotides Directed at Exon 39

Antisense oligonucleotides directed at exon 39 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H39A(+62+85) [SEQ ID NO:153] induced exon 39 skipping when delivered into cells at a concentration of 100 nM. Table 33 below shows the antisense molecules tested and their ability to induce exon skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H39A(+62+85)	CUG GCU UUC UCU CAU CUG UGA UUC	Skipping to 100 nM
H39A(+39+58)	GUU GUA AGU UGU CUC CUC UU	No skipping
H39A(+102+121)	UUG UCU GUA ACA GCU GCU GU	No skipping
H39D(+10-10)	GCU CUA AUA CCU UGA GAG CA	Skipping to 300 nM

Table 33

Antisense Oligonucleotides Directed at Exon 40

Antisense oligonucleotides directed at exon 40 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 19 illustrates antisense molecule H40A(-05+17) [SEQ ID NO:157] directed at exon 40 acceptor splice site. H40A(-05+17) and H40A(+129+153) [SEQ ID NO:158] both substantially induced exon 40 skipping when delivered into cells at a concentration of 5 nM.

Antisense Oligonucleotides Directed at Exon 42

Antisense oligonucleotides directed at exon 42 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 20 illustrates antisense molecule H42A(-04+23) [SEQ ID NO:159], directed at exon 42 acceptor splice site. H42A(-4+23) and H42D(+19-02) [SEQ

ID NO:161] both induced exon 42 skipping when delivered into cells at a concentration of 5 nM. Table 34 below shows the antisense molecules tested and their ability to induce exon 42 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H42A(-4+23)	AUC GUU UCU UCA CGG ACA GUG UGC UGG	Skipping to 5 nM
H42A(+86+109)	GGG CUU GUG AGA CAU GAG UGA UUU	Skipping to 100 nM
H42D(+19-02)	A CCU UCA GAG GAC UCC UCU UGC	Skipping to 5 nM

Table 34

Antisense Oligonucleotides Directed at Exon 43

Antisense oligonucleotides directed at exon 43 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H43A(+101+120) [SEQ ID NO:163] induced exon 43 skipping when delivered into cells at a concentration of 25 nM. Table 35 below includes the antisense molecules tested and their ability to induce exon 43 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H43D(+10-15)	UAU GUG UUA CCU ACC CUU GUC GGU C	Skipping to 100 nM
H43A(+101+120)	GGA GAG AGC UUC CUG UAG CU	Skipping to 25 nM
H43A(+78+100)	UCA CCC UUU CCA CAG GCG UUG CA	Skipping to 200 nM

Table 35

Antisense Oligonucleotides Directed at Exon 44

Antisense oligonucleotides directed at exon 44 were prepared using similar methods as described above. Testing for the ability of these antisense molecules to induce exon 44 skipping is still in progress. The antisense molecules under review are shown as SEQ ID Nos: 165 to 167 in Table 1A.

Antisense Oligonucleotides Directed at Exon 45

Antisense oligonucleotides directed at exon 45 were prepared using similar methods as described above. Testing for the ability of these antisense molecules to induce exon 45 skipping is still in progress. The antisense molecules under review are shown as SEQ ID Nos: 207 to 211 in Table 1A.

Antisense Oligonucleotides Directed at Exon 46

Antisense oligonucleotides directed at exon 46 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 21 illustrates the efficiency of one antisense molecule directed at exon 46 acceptor splice site. Antisense oligonucleotide H46A(+86+115) [SEQ ID NO:203] showed very strong ability to induce exon 46 skipping. Table 36 below includes antisense molecules tested. These antisense molecules showed varying ability to induce exon 46 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H46D(+16-04)	UUA CCU UGA CUU GCU CAA GC	No skipping
H46A(+90+109)	UCC AGG UUC AAG UGG GAU AC	No skipping
H46A(+86+115)	CUC UUU UCC AGG UUC AAG UGG GAU ACU AGC	Good skipping to 100 nM
H46A(+107+137)	CAA GCU UUU CUU UUA GUU GCU GCU CUU UUC C	Good skipping to 100 nM
H46A(-10+20)	UAU UCU UUU GUU CUU CUA GCC UGG AGA AAG	Weak skipping
H46A(+50+77)	CUG CUU CCU CCA ACC AUA AAA CAA AUU C	Weak skipping

Table 36

Antisense Oligonucleotides Directed at Exon 47

Antisense oligonucleotides directed at exon 47 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

H47A(+76+100) [SEQ ID NO:170] and H47A(-09+12) [SEQ ID NO:172] both induced exon 47 skipping when delivered into cells at a concentration of 200 nM. H47D(+25-02) [SEQ ID NO: 171] is yet to be prepared and tested.

Antisense Oligonucleotides Directed at Exon 50

Antisense oligonucleotides directed at exon 50 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Antisense oligonucleotide molecule H50(+02+30) [SEQ ID NO: 173] was a strong inducer of exon skipping. Further, H50A(+07+33) [SEQ ID NO:174] and H50D(+07-18) [SEQ ID NO:175] both induced exon 50 skipping when delivered into cells at a concentration of 100 nM.

Antisense Oligonucleotides Directed at Exon 51

Antisense oligonucleotides directed at exon 51 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 22 illustrates differing efficiencies of two antisense molecules directed at exon 51 acceptor splice site. Antisense oligonucleotide H51A(+66+90) [SEQ ID NO:180] showed the stronger ability to induce exon 51 skipping. Table 37 below includes antisense molecules tested at a concentration range of 25, 50, 100, 300 and 600 nM. These antisense molecules showed varying ability to induce exon 51 skipping. The strongest inducers of exon skipping were antisense oligonucleotide H51A(+61+90) [SEQ ID NO: 179] and H51A(+66+95) [SEQ ID NO: 179].

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H51A(-01+25)	ACC AGA GUA ACA GUC UGA GUA GGA GC	Faint skipping
H51D(+16-07)	CUC AUA CCU UCU GCU UGA UGA UC	Skipping at 300 nM
H51A(+111+134)	UUC UGU CCA AGC CCG GUU GAA AUC	Needs re-testing
H51A(+61+90)	ACA UCA AGG AAG AUG GCA UUU CUA GUU	Very strong

Antisense oligonucleotide name	Sequence	Ability to induce skipping
	UGG	skipping
H51A(+66+90)	ACA UCA AGG AAG AUG GCA UUU CUA G	skipping
H51A(+66+95)	CUC CAA CAU CAA GGA AGA UGG CAU UUC UAG	Very strong skipping
H51D(+08-17)	AUC AUU UUU UCU CAU ACC UUC UGC U	No skipping
H51A/D(+08-17) & (-15+?)	AUC AUU UUU UCU CAU ACC UUC UGC UAG GAG CUA AAA	No skipping
H51A(+175+195)	CAC CCA CCA UCA CCC UCY GUG	No skipping
H51A(+199+220)	AUC AUC UCG UUG AUA UCC UCA A	No skipping

Table 37

Antisense Oligonucleotides Directed at Exon 52

Antisense oligonucleotides directed at exon 52 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 22 also shows differing efficiencies of four antisense molecules directed at exon 52 acceptor splice site. The most effective antisense oligonucleotide for inducing exon 52 skipping was H52A(+17+37) [SEQ ID NO:188].

Table 38 below shows antisense molecules tested at a concentration range of 50, 100, 300 and 600 nM. These antisense molecules showed varying ability to induce exon 50 skipping. Antisense molecules H52A(+12+41) [SEQ ID NO:187] and H52A(+17+37) [SEQ ID NO:188] showed the strongest exon 50 skipping at a concentration of 50 nM.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H52A(-07+14)	UCC UGC AUU GUU GCC UGU AAG	No skipping
H52A(+12+41)	UCC AAC UGG GGA CGC CUC UGU UCC AAA UCC	Very strong skipping
H52A(+17+37)	ACU GGG GAC GCC UCU GUU CCA	Skipping to 50 nM
H52A(+93+112)	CCG UAA UGA UUG UUC UAG CC	No skipping
H52D(+05-15)	UGU UAA AAA ACU UAC UUC GA	No skipping

Table 38

Antisense Oligonucleotides Directed at Exon 53

Antisense oligonucleotides directed at exon 53 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

Figure 22 also shows antisense molecule H53A(+39+69) [SEQ ID NO:193] directed at exon 53 acceptor splice site. This antisense oligonucleotide was able to induce exon 53 skipping at 5, 100, 300 and 600 nM. A "cocktail" of three exon 53 antisense oligonucleotides:- H53D(+23+47) [SEQ ID NO:195], H53A(+150+175) [SEQ ID NO:196] and H53A(+14-07) [SEQ ID NO:194], were also tested, as shown in Figure 20 and exhibited an ability to induce exon skipping.

Table 39 below includes other antisense molecules tested at a concentration range of 50, 100, 300 and 600 nM. These antisense molecules showed varying ability to induce exon 53 skipping. Antisense molecule H53A(+39+69) [SEQ ID NO:193] induced the strongest exon 53 skipping.

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H53A(+45+69)	CAU UCA ACU GUU GCC UCC GGU UCU G	Faint skipping at 50 nM
H53A(+39+62)	CUG UUG CCU CCG GUU CUG AAG GUG	Faint skipping at 50 nM
H53A(+39+69)	CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G	Strong skipping to 50 nM
H53D(+14-07)	UAC UAA CCU UGG UUU CUG UGA	Very faint skipping to 50 nM
H53A(+23+47)	CUG AAG GUG UUC UUG UAC UUC AUC C	Very faint skipping to 50 nM
H53A(+150+176)	UGU AUA GGG ACC CUC CUU CCA UGA CUC	Very faint skipping to 50 nM
H53D(+20-05)	CUA ACC UUG GUU UCU GUG AUU UUC U	Not made yet
H53D(+09-18)	GGU AUC UUU GAU ACU AAC CUU GGU UUC	Faint at 600 nM
H53A(-12+10)	AUU CUU UCA ACU AGA AUA AAA G	No skipping
H53A(-07+18)	GAU UCU GAA UUC UUU CAA CUA GAA U	No skipping
H53A(+07+26)	AUC CCA CUG AUU CUG AAU UC	No skipping

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H53A(+124+145)	UUG GCU CUG GCC UGU CCU AAG A	No skipping

Table 39

The Claims Defining the Invention are as Follows

1. An antisense molecule capable of binding to a selected target site to induce exon skipping in the dystrophin gene, as set forth in SEQ ID NO: 1 to 202.

2. An antisense molecule according to claim 1 capable of inducing exon skipping in exons 3, 4, 8, 10 to 16, 19 to 40, 42 to 44, 46, 47 and 50 to 53 of the dystrophin gene.

3. A combination of two or more antisense molecules according to claim 1 or 2 capable of binding to a selected target to induce exon skipping in the dystrophin gene.

4. A combination or two or more antisense molecules according to claim 3 selected from Table 1B.

5. A combination of two or more antisense molecules according to claim 1 or 2 joined together to form a "weasel", wherein said weasel is capable of binding to a selected target to induce exon skipping in the dystrophin gene.

6. A combination of two or more antisense molecules according to claim 5 selected from Table 1C.

7. The antisense molecule according to any one of claims 1 to 6, capable of binding to a selected target site, wherein the target site is an mRNA splicing site selected from a splicer donor site, splice acceptor sites or exonic splicing enhancer elements.

8. A method of treating muscular dystrophy in a patient comprising administering to the patient a composition comprising an antisense molecule according to anyone of claims 1 to 6.

9. A pharmaceutical or therapeutic composition for the treatment of muscular dystrophy in a patient comprising (a) at least an antisense molecule

according to any one of claims 1 to 6, and (b) one or more pharmaceutically acceptable carriers and/or diluents.

10. The composition according to claim 9, comprising about 20 nM to 600 nM of the antisense molecule.

11. The use of an antisense molecule according to any one of claims 1 to 6 for the manufacture of a medicament for modulation of muscular dystrophy.

12. An antisense molecule according to any one of claims 1 to 6 for use in antisense molecule based therapy.

13. An antisense molecule according to any one of claims 1 to 6 as herein before described with reference to the examples.

14. A kit comprising at least one antisense molecule according to any one of claims 1 to 6, a suitable carrier and instructions for its use.

FIGURE 1.

bp Acceptor ESE Donor

ucaugcacugagugaccucuuucucgcagGCGCUAGCUGGAGCA/////CCGUGCAGACUGACGgucucau

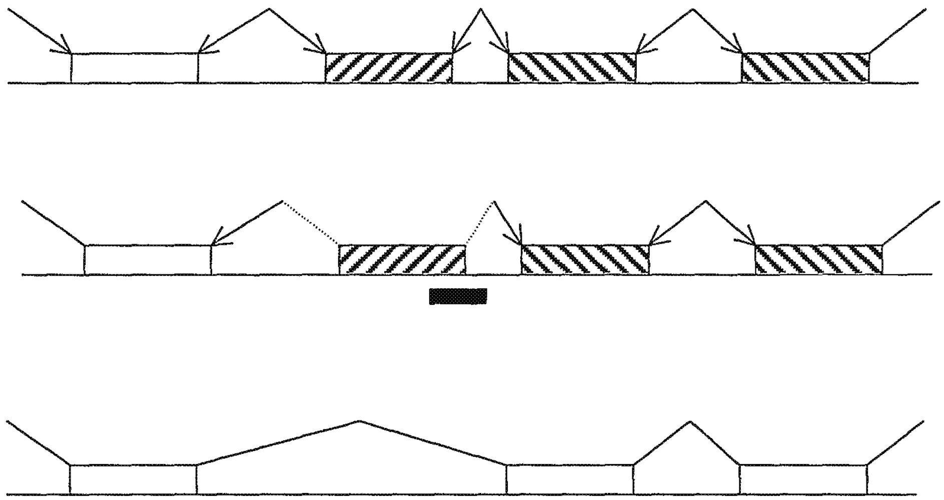


FIGURE 2

H8A(-06+14) | H8A(-06+18)

M 600 300 100 50 20 UT 600 300 100 50 20 UT M

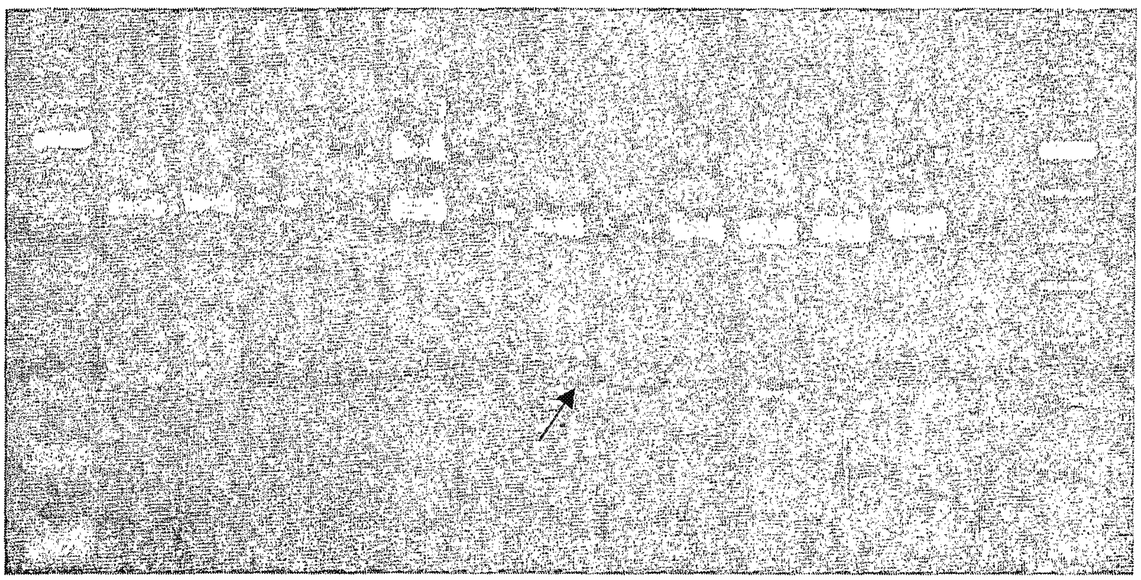


FIGURE 3

H7A(+45+67) H7A(+2+26)

M 600 300 100 50 20 600N M 600 300 100 50 20 600N M

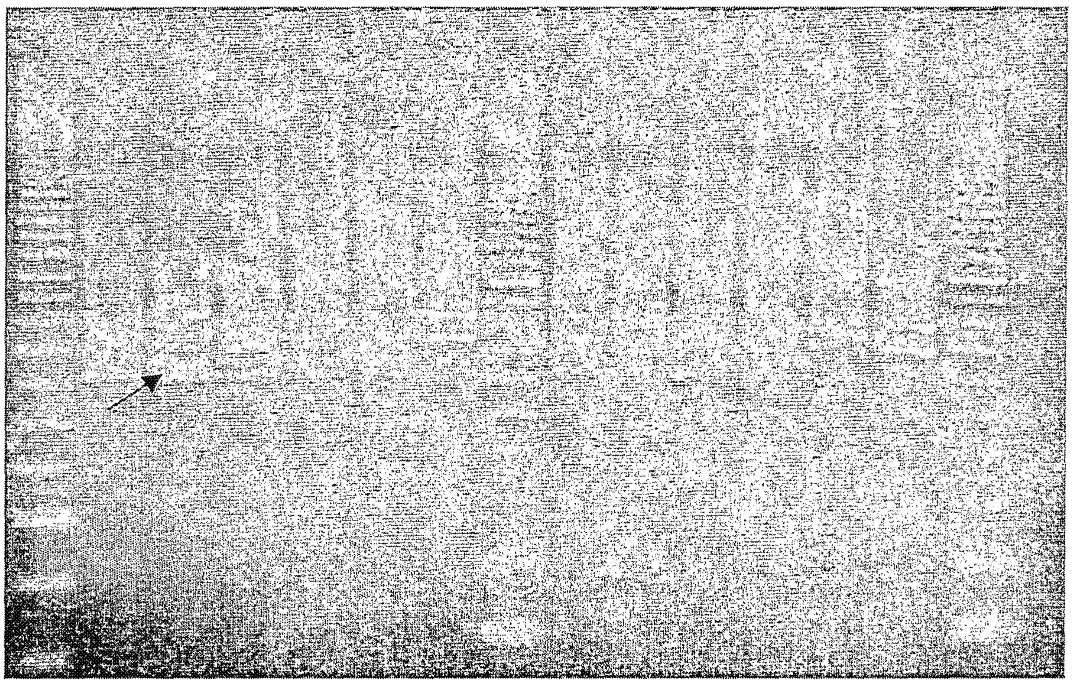


FIGURE 4

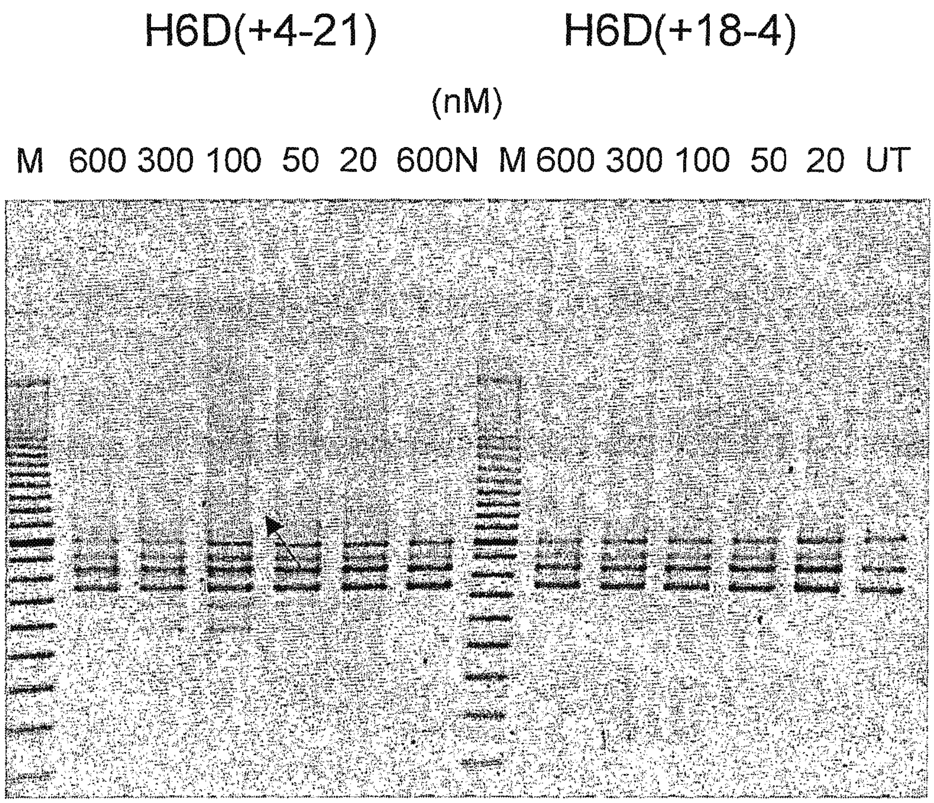


FIGURE 5

6A(+69+91)

M 600 300 200 100 50 20 UT

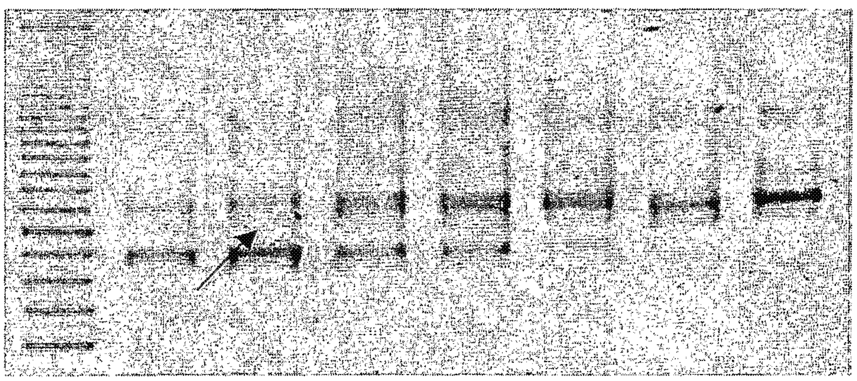


FIGURE 6

H4A(+13+32)

M 600 300 100 50 20 UT Neg M

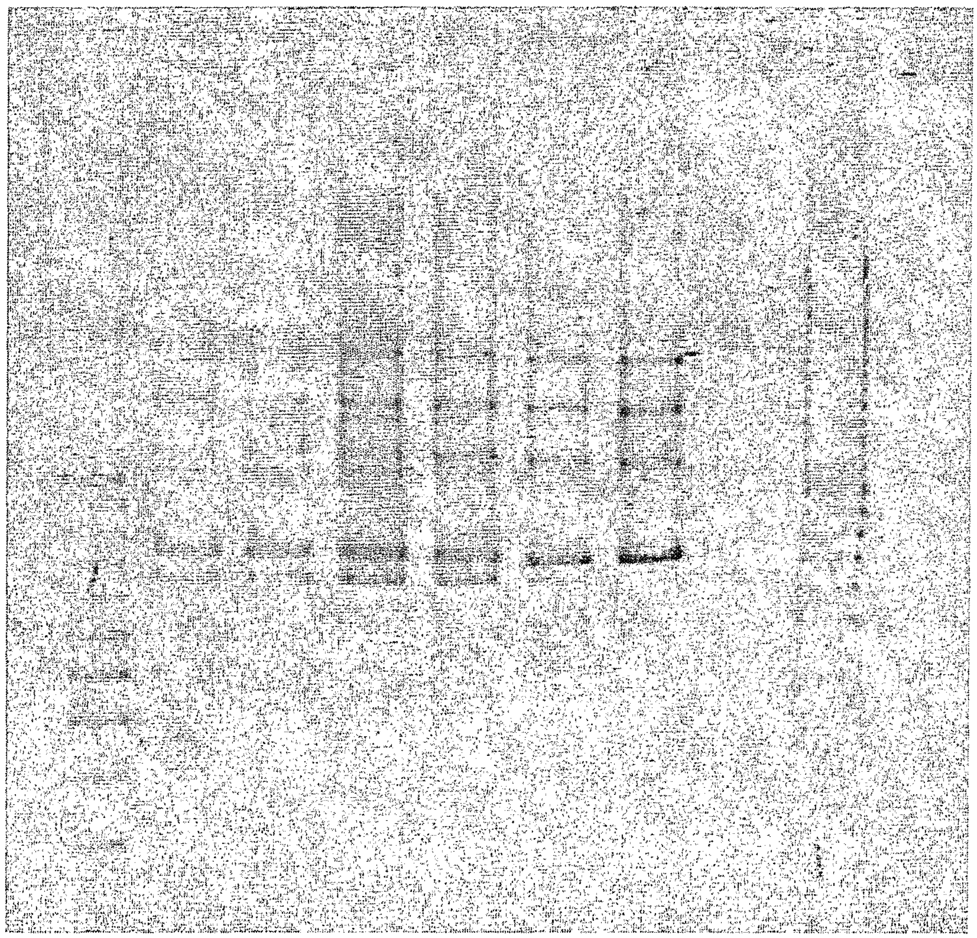
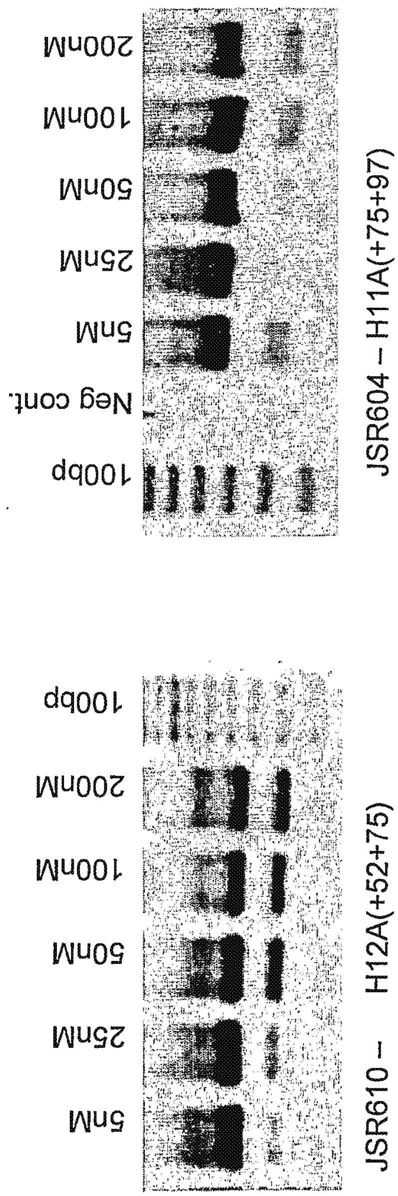


FIGURE 7


5nM
25nM
50nM
100nM
200nM
100bp
JSR610 – H12A(+52+75)
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Neg cont.
5nM
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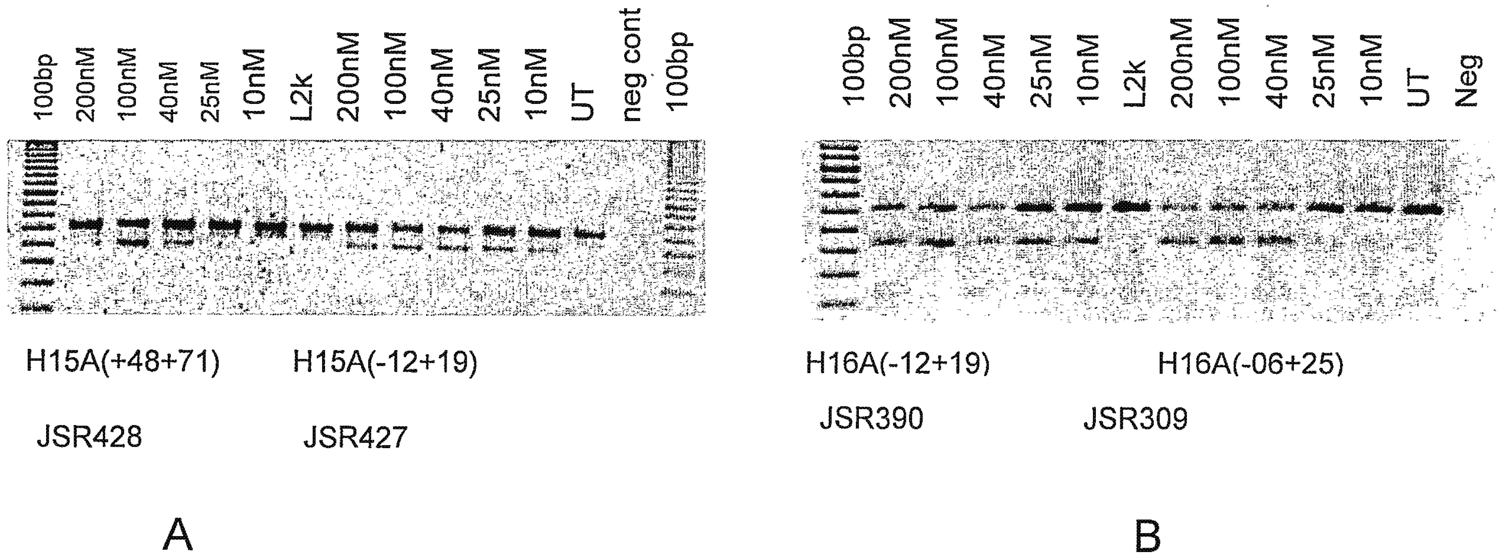
50nM
100nM
200nM
JSR604 – H11A(+75+97)

A

B

FIGURE 8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100nM
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L2k
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100nM
40nM
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H15A(-12+19)
JSR428
JSR427
A
100bp
200nM
100nM
40nM
25nM
10nM
L2k
200nM
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UT
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H16A(-12+19)
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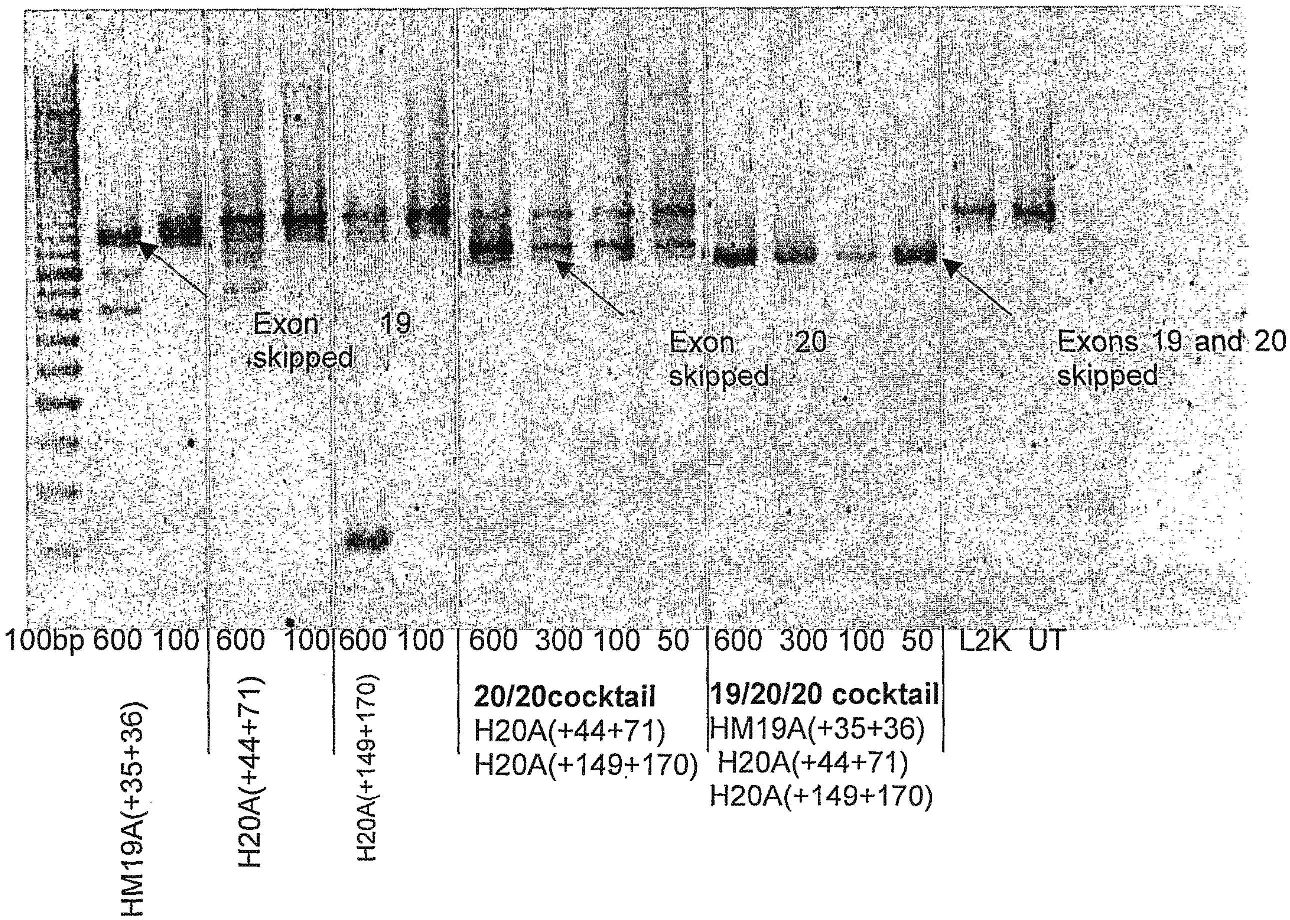
JSR390
JSR309
B

FIGURE 9


Exon 19 skipped
Exon 20 skipped
Exons 19 and 20 skipped
100bp 600 100 600 100 600 100 600 300 100 50 600 300 100 50 L2K UT
HM19A(+35+36)
H20A(+44+71)
H20A(+149+170)
20/20cocktail
H20A(+44+71)
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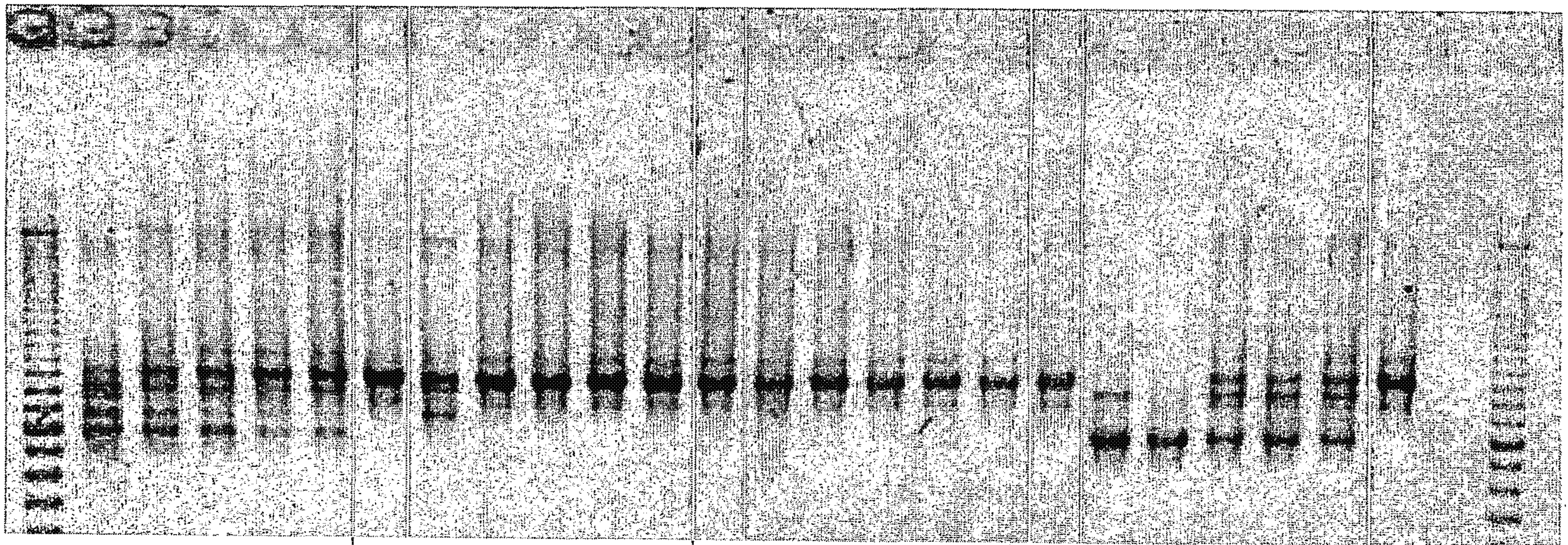
19/20/20 cocktail
HM19A(+35+36)
H20A(+44+71)
H20A(+149+170)

FIGURE 10



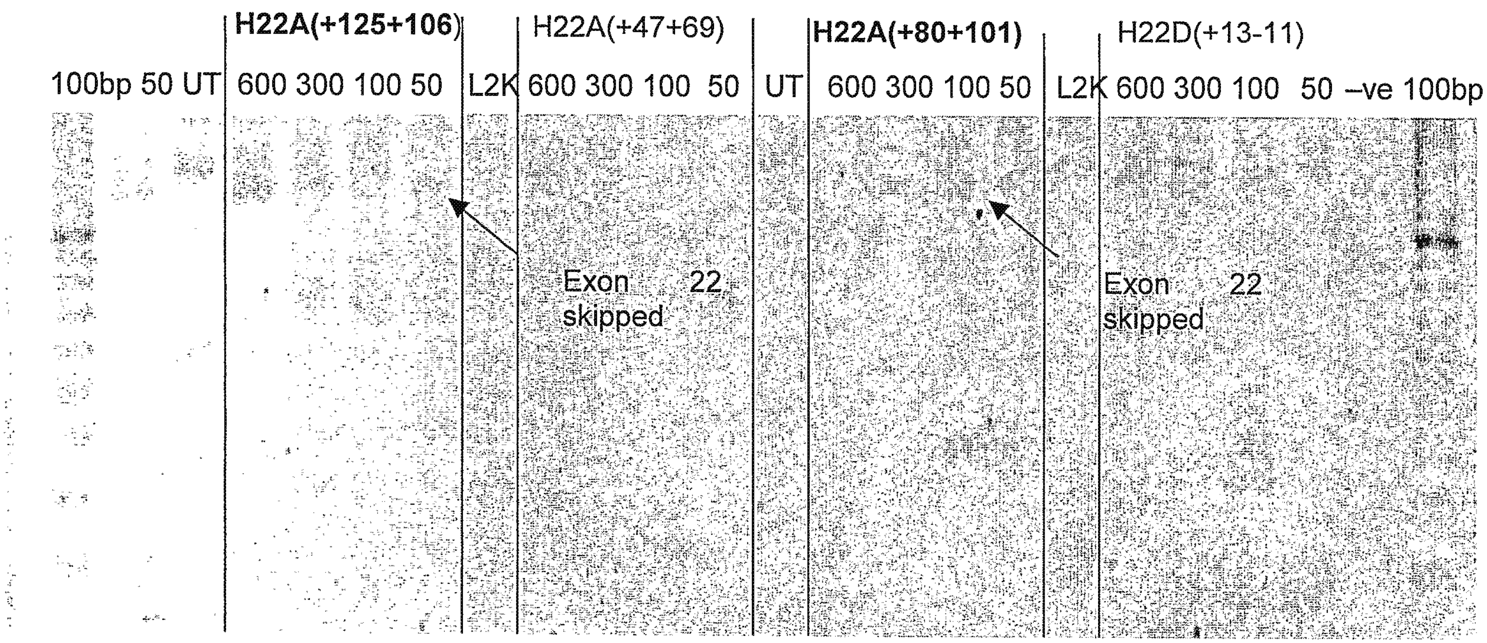
Weasel 19/20/20
H19A(+35+53)-aa-
H20A(+44+63)-aa-
H20A(+149+168)

Weasel19/20
H19A(+35+53)-
aa-
H20A(+44+63

Weasel19/20
H19A(+35+53)-
aa-
H20A(+149+168)

19/20/20 cocktail
HM19A(+35+36)
H20A(+44+71)
H20A(+149+170)

FIGURE 11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H22A(+47+69)
H22A(+80+101)
H22D(+13-11)
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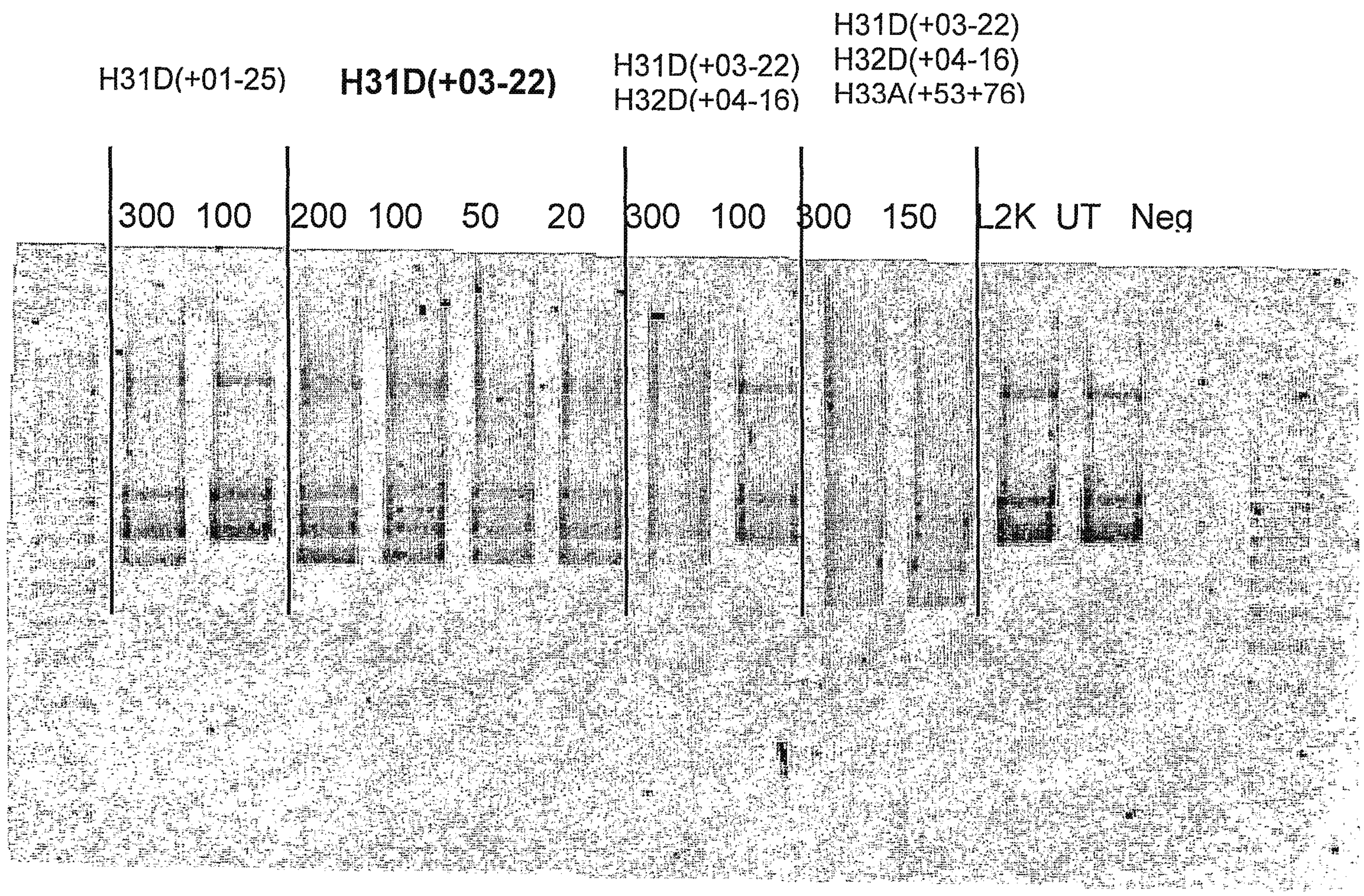
Exon 22 skipped
Exon 22 skipped

FIGURE 12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H31D(+03-22)
H31D(+03-22)
H32D(+04-16)
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H33A(+53+76)
300 100 200 100 50 20 300 100 300 150 L2K UT Neg

FIGURE 13

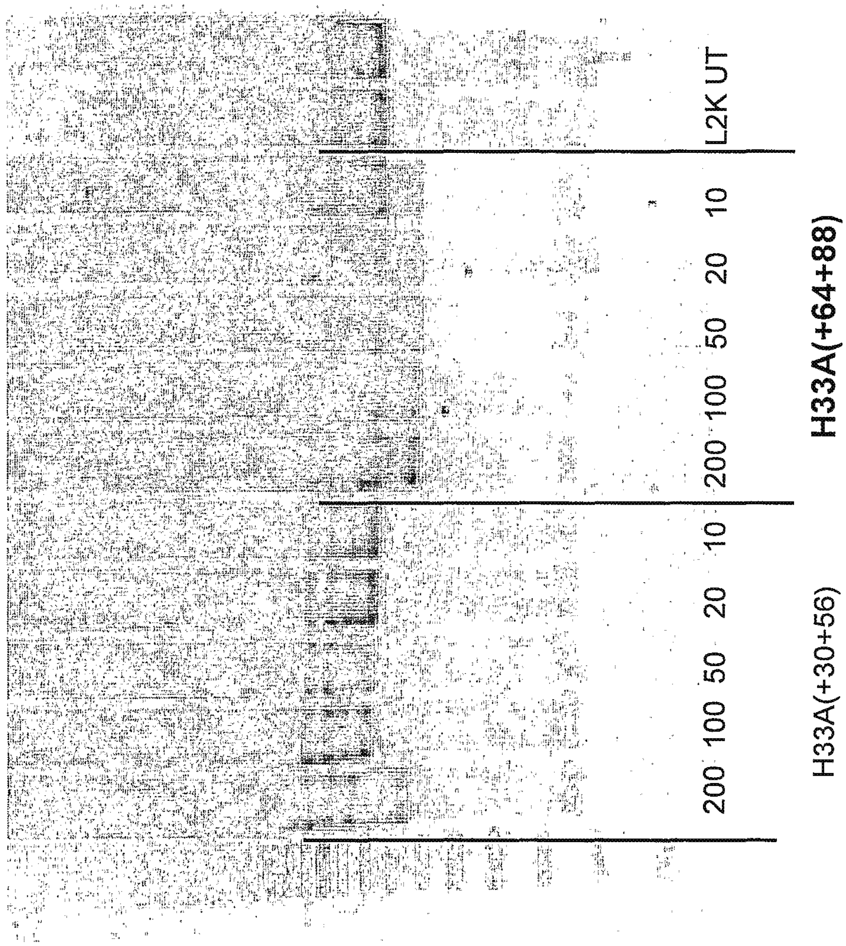
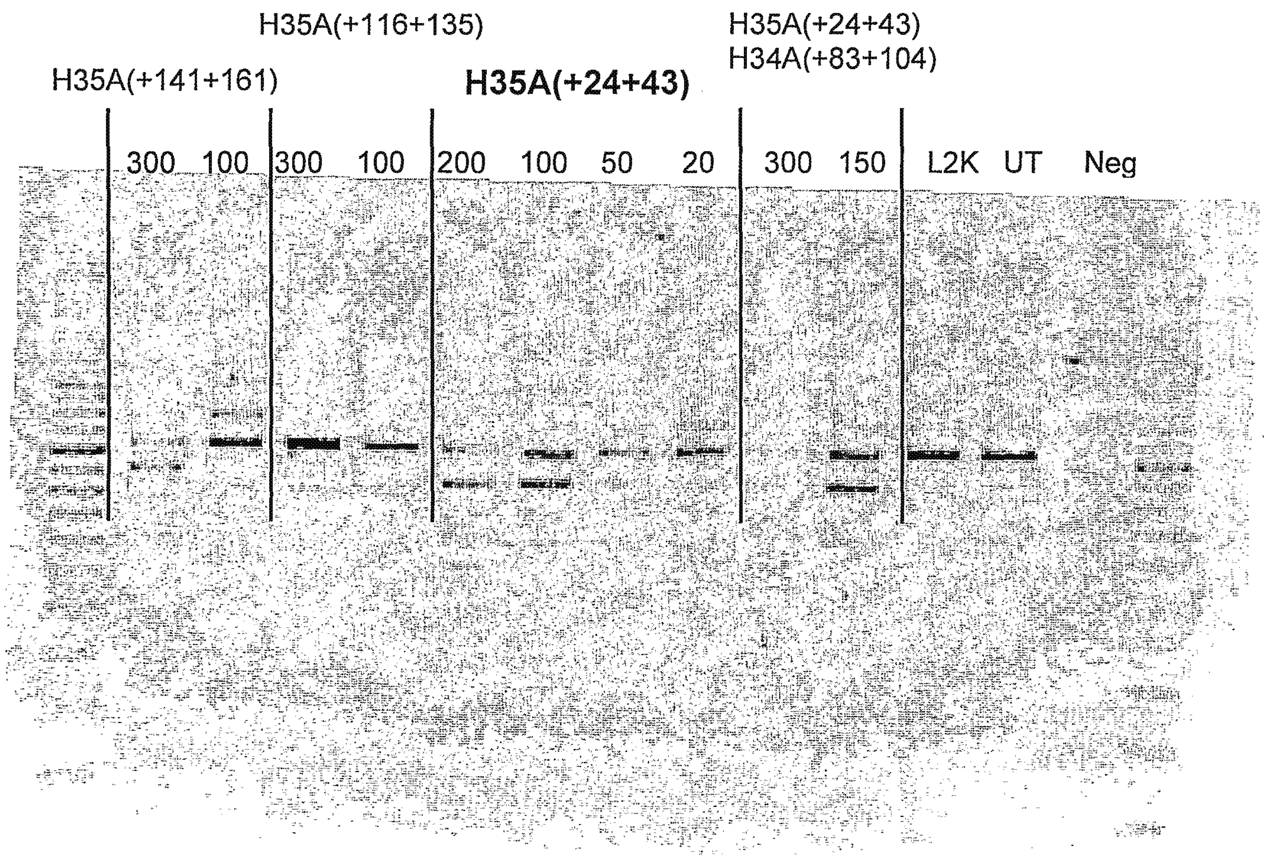


FIGURE 14



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H35A(+24+43)
H34A(+83+104)
H35A(+141+161)
H35A(+24+43)
300 100 300 100 200 100 50 20 300 150 L2K UT Neg

FIGURE 15

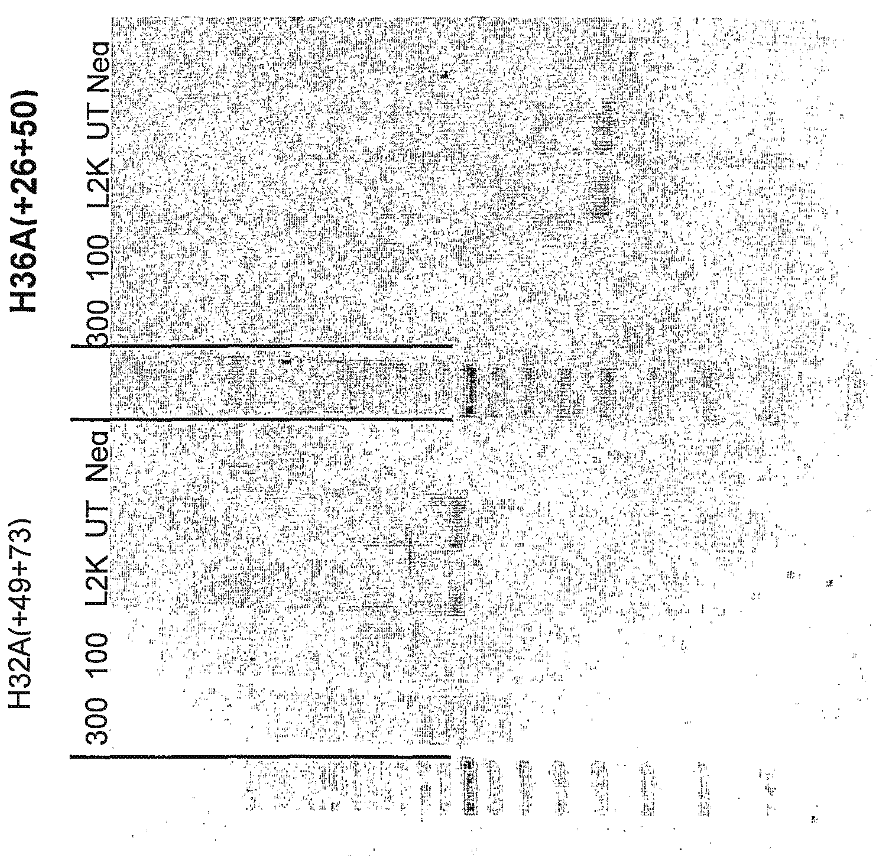


FIGURE 16

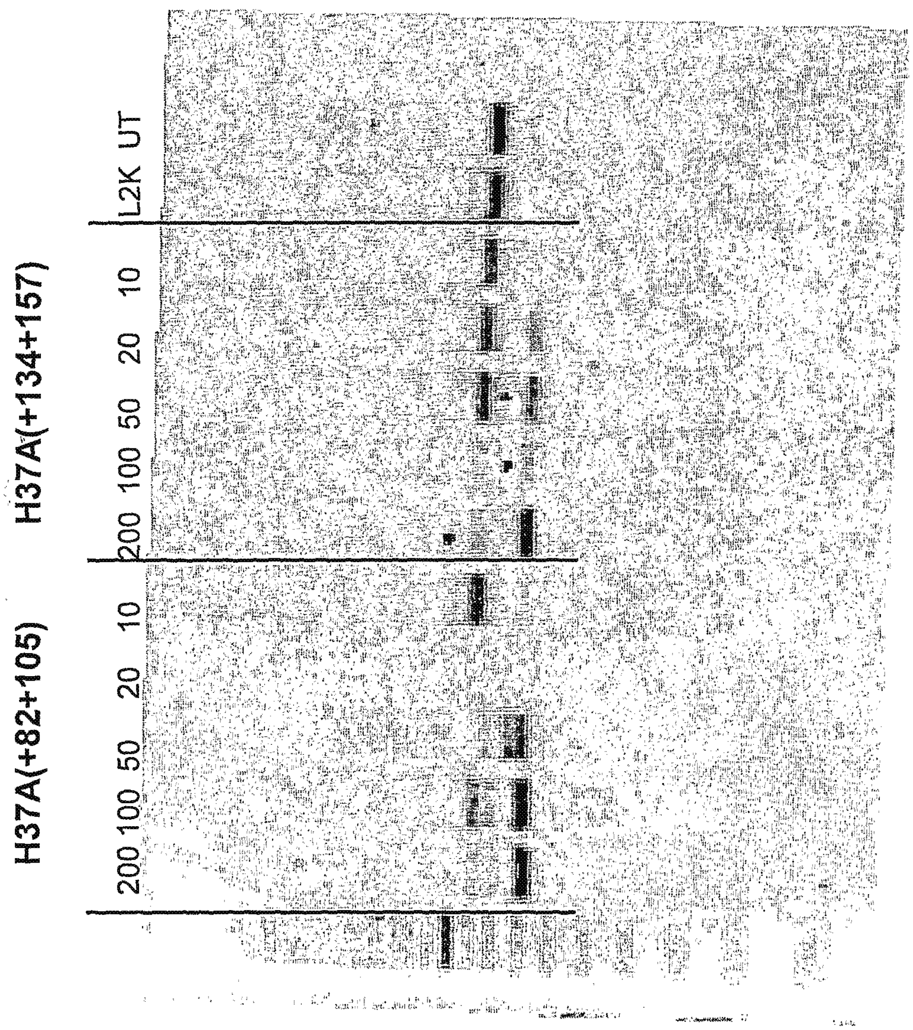


FIGURE 17

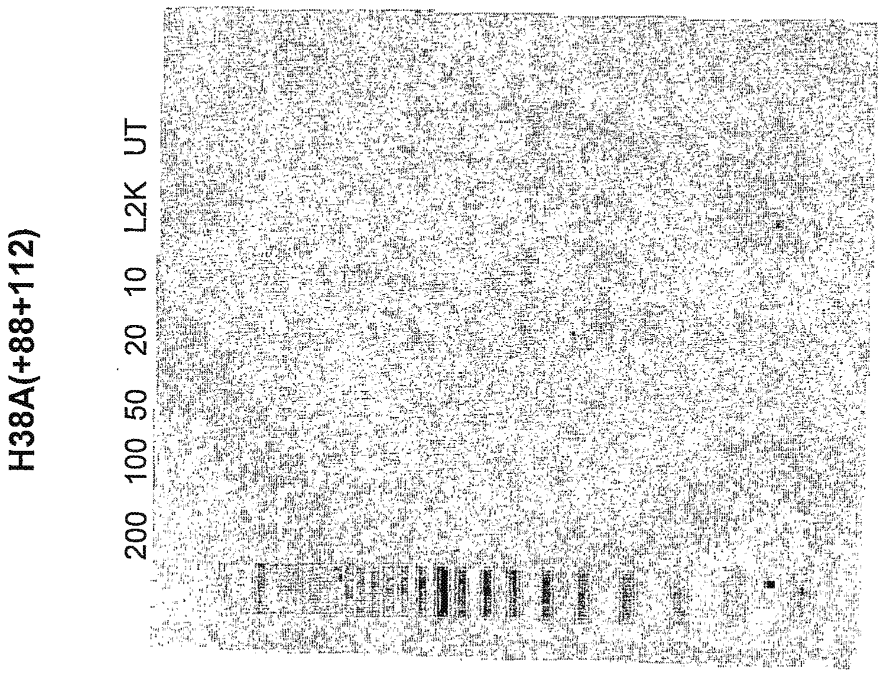


FIGURE 18

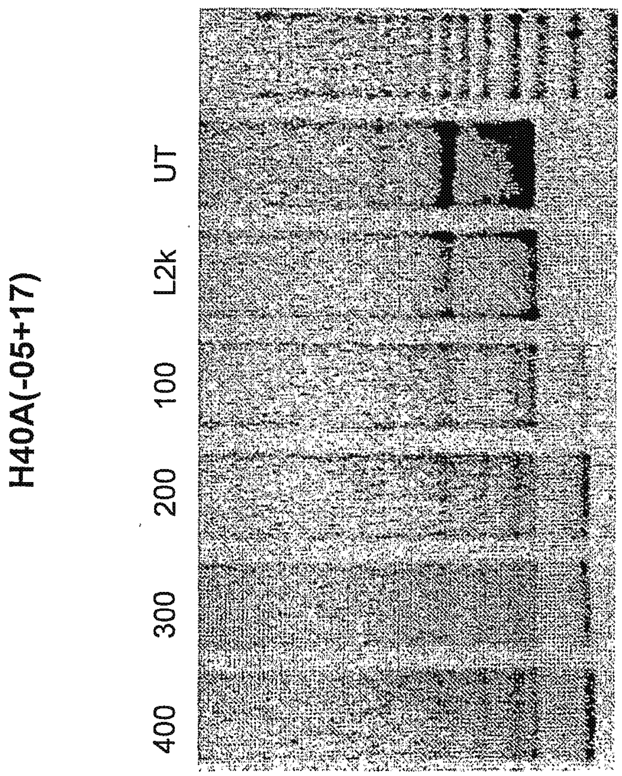


FIGURE 19

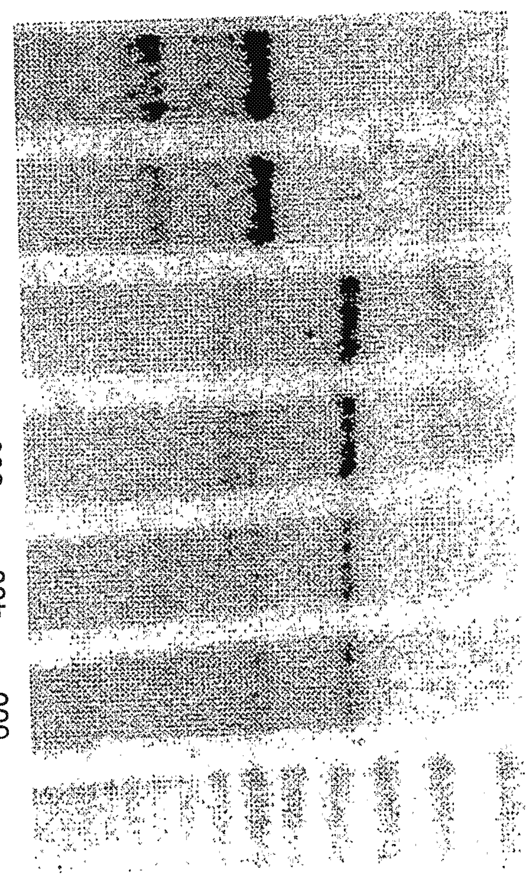


FIGURE 20

H46A(+86+115)

600 300 200 100 L2K UT

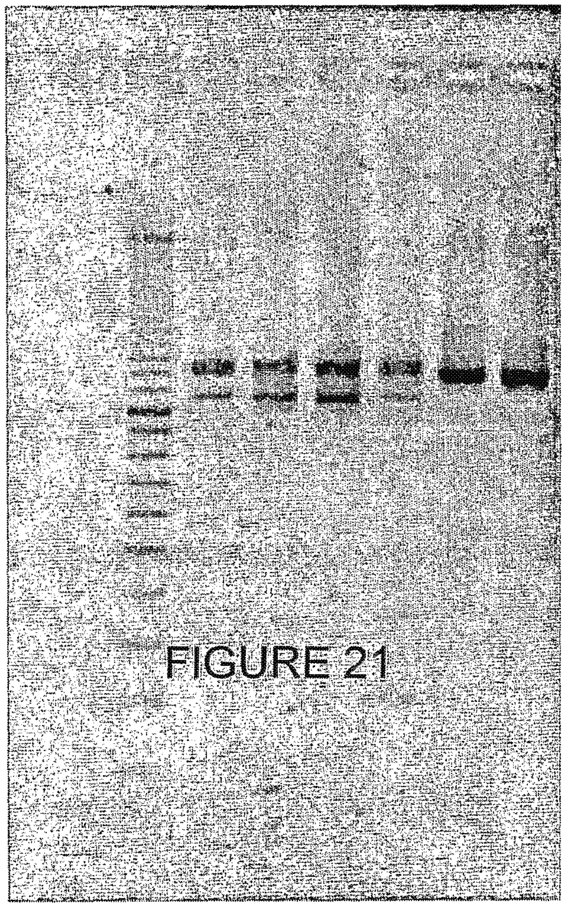
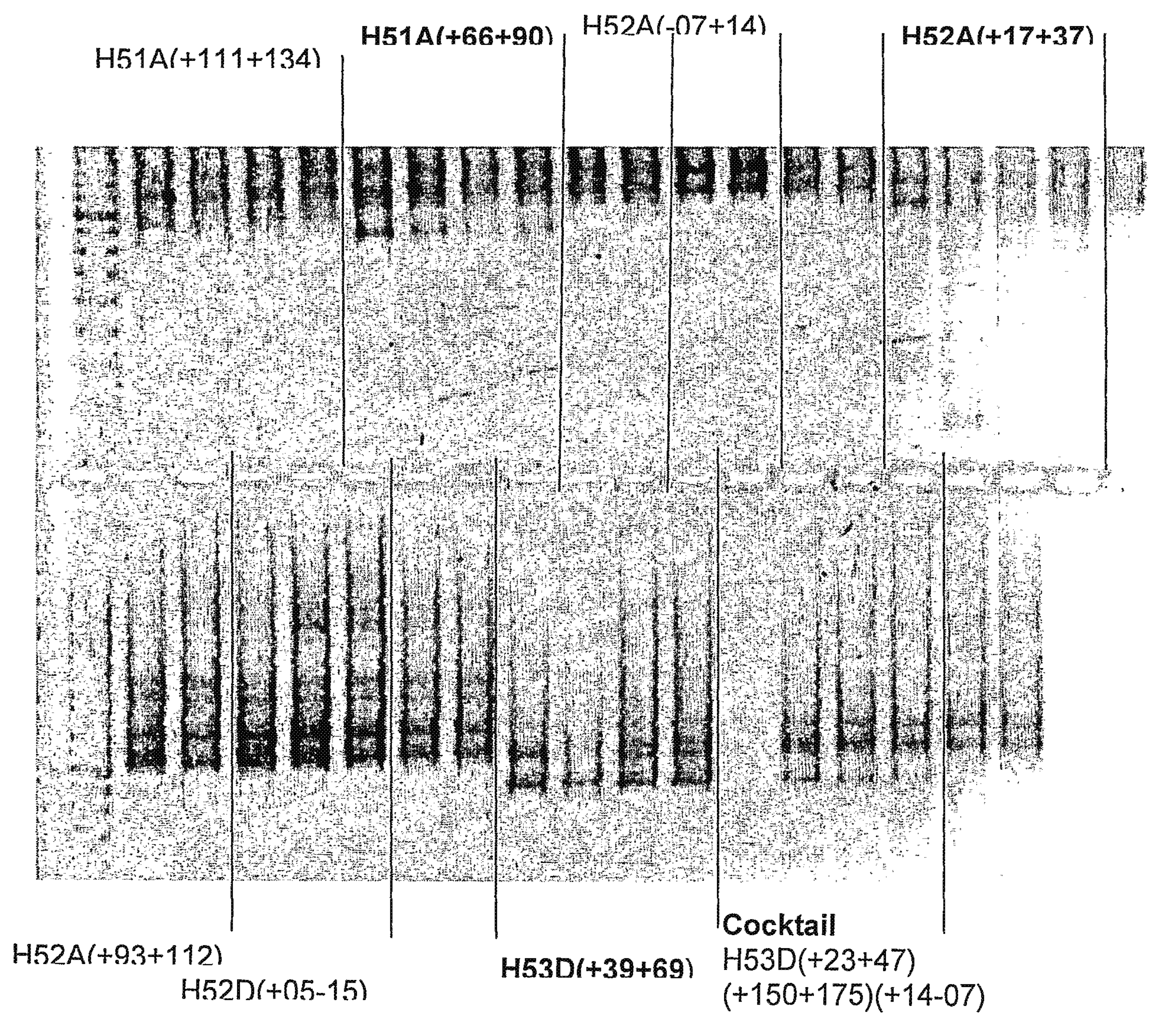


FIGURE 21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H51A(+66+90)
H52A(-07+14)
H52A(+17+37)
H52A(+93+112)
H52D(+05-15)
H53D(+39+69)
Cocktail
H53D(+23+47)
(+150+175)(+14-07)

FIGURE 22

115361Sequence_listing_final_1.ST25.ST25
SEQUENCE LISTING

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<120> Antisense Oligonucleotides for Inducing Exon Skipping and Methods of Use Thereof.

<130> 111819

<160> 211

<170> PatentIn version 3.3

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/000943

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. 7: C12N 15/11 A61K 48/00, 31/7088, 31/712 C07H 21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS, MEDLINE, CAPLUS : exon skip, DMD, dystrophin, muscular dystrophy
GENBANK, DGENE: SEQ ID NOs:1, 181, 193

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AU 2003284638 A1 (KOBE UNIVERSITY & SANKYO COMPANY, LIMITED) 18 June 2004, pages 264-291.	1-14
X	WO 2002/024906 A1 (ACADEMISCH ZIEKENHUIS LEIDEN) 28 March 2002, pages 21-23.	1-14
X	AU 780517 B2 (JCR PHARMACEUTICALS CO., LTD.) 1 November 2001, whole document.	1-14

[X] Further documents are listed in the continuation of Box C   [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 September 2005	20 OCT 2005
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **LEXIE PRESS** Telephone No : (02) 6283 2677

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/000943

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AARTSMA-RUS, A et al (2004) 'Antisense-induced multiexon skipping for duchenne muscular dystrophy makes more sense'. American Journal of Human Genetics [online], Vol 74 (1): pages 83-92, [retrieved on 5 October 2005]. Retrieved from: <URL: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1181915>, the whole document	1-14
X	DE ANGELIS F.G. et al (2002) 'Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Δ 48-50 DMD cells'. Proceedings of the National Academy of Sciences (USA), Vol 99(14): pages 9456-9461, the whole document.	1-14
X	AARTSMA-RUS, A. et al (2002) 'Targeted exon skipping as a potential gene correction therapy for duchenne muscular dystrophy'. Neuromuscular Disorders, Vol 12(Suppl.): pages S71-S77, page S73	1-14
X	AARTSMA-RUS, A. et al (2003) 'Therapeutic antisense-induced exon skipping in cultures muscle cells from six different DMD patients'. Human Molecular Genetics, Vol 12(8): pages 907-914, the whole document.	1-14
P, X	WO 2004/083432 A1 (ACADEMISCH ZIEKENHUIS LEIDEN) 30 September 2004, pages 47-48	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/000943

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box I

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to: **the claims as they relate to SEQ ID NOs: 1, 181, and 193 (See supplemental Box I).**

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/000943

Supplemental Box I
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: III

The international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.
Note that Rule 13.2 states that where a group of inventions is claimed in one and the same international application, the requirement of unity of invention referred to in Rule 13.1 shall be fulfilled only where there is a technical relationship among those inventions involving one or more of the same corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

The ISA has identified 202 separate inventions.

Claim 1 and dependent claims are directed to 202 different antisense molecules that are capable of inducing exon skipping in the dystrophin gene.

Although all of the sequences share the feature that they are capable of inducing exon skipping in the dystrophin gene, this does not represent a special technical feature.

This feature cannot be a special technical feature because it is not novel. Antisense induced exon skipping in the dystrophin gene is disclosed in a number of documents, including:

Aartsma-Rus, A. et al (2004) American Journal of Human Genetics [online], Vol 74: 83-92

WO 2002/024906 A1 (ACADEMISCH ZIEKENHUIS LEIDEN) 28.03.2002.

AU 780517 B2 (JCR PHARMACEUTICALS CO., LTD.) 01.11.2001.

De Angelis, F.G. et al (2002) Proceedings of the National Academy of Sciences (USA), Vol 99(14): 9456-9461.

Furthermore, given the nature of the claims and the invention, it is appropriate to apply the Markush approach. Although the 202 sequences all share a common property and represent a single recognised class ie. all are derived from the dystrophin gene, this class of sequences is known (see above citations). Thus, according to the Markush approach, these sequences do not represent a single invention.

After communications with the applicant it was agreed that the ISA would search three inventions (SEQ ID NOs: 1, 181, and 193) for the one search fee already paid. Accordingly, claims 1-14 as they relate to SEQ ID NOs: 1, 181, and 193, have been searched.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2005/000943

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2003284638	WO	2004048570	CA	2507125	EP	1568769
WO	200224906	AU	11062/02	CA	2423044	EP	1191097
		EP	1320597	NZ	524853	US	2003235845
AU	780517	AU	61354/00	CA	2319149	EP	1160318
		EP	1544297	JP	2002010790	US	6653467
WO	2004083432	AU	2003225410	WO	2004083446		

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX

EXHIBIT 12

IN THE UNITED STATES DISTRICT COURT
DISTRICT OF DELAWARE

NIPPON SHINYAKU CO., LTD., Plaintiff, v. SAREPTA THERAPEUTICS, INC., Defendant.	)	C.A. No. 21-1015 (GBW)
SAREPTA THERAPEUTICS, INC., and THE UNIVERSITY OF WESTERN AUSTRALIA Counterclaimants v. NIPPON SHINYAKU CO., LTD. and NS PHARMA, INC., Counterclaim Defendants.	)	

NIPPON SHINYAKU CO. LTD. AND NS PHARMA, INC.'S NONINFRINGEMENT CONTENTIONS

Pursuant to Paragraph 3(g) of the of the Scheduling Order (D.I. 143), Plaintiff/Counterclaim Defendant Nippon Shinyaku Co. Ltd. and Counterclaim Defendant NS Pharma, Inc. (collectively "NS") provide the following noninfringement contentions for each asserted claim to Defendant/Counter-Plaintiff Sarepta Therapeutics, Inc. ("Sarepta"). These Noninfringement Contentions disclose the current bases for Defendants' contentions regarding noninfringement.

NS provides this disclosure based on the information and evidence available to it at this time, without the benefit of full discovery. NS therefore reserves its right to make any modifications, additions, deletions, or supplementations to this disclosure as additional evidence and information become available, or as is otherwise appropriate and permissible.

I. The UWA Patents

These contentions relate to the claims that Sarepta asserts in its June 22, 2023 Infringement Contentions against NS U.S. Patent Nos. 9,994,851 ("the '851 patent"); 10,227,590 ("the '590 patent"); and 10,266,827 ("the '827 patent") (collectively, "the UWA Patents"). Sarepta's infringement contentions assert claims 1 and 2 of the '851 Patent, claims 1 and 2 of the '590 Patent, and claims 1 and 2 of the '827 Patent (collectively, the "Asserted Claims").

To the extent Sarepta is granted leave to amend its contentions to add any claims from these or other patents, NS will serve supplemental contentions directed at any such additional claims.

These contentions are based on NS's present understanding of the asserted claims and/or claim constructions that may be discerned in light of Sarepta's allegations of infringement and apparent interpretation of the asserted claims. NS's contentions are not, and should in no way be interpreted as, admissions, suggestions, or adoptions of any particular claim scope or construction. NS reserves the right to amend or supplement its contentions to address any further information provided by the Court or Sarepta regarding the meaning or scope of the terms of the asserted claims.

II. Sarepta's Infringement Contentions

Sarepta has claimed that "[b]y making, using, selling, offering to sell, and/or importing Viltepso® (viltolarsen), NS **directly and indirectly** infringes the asserted claims of the Wilton Patents [i.e., the UWA patents]." Final Infringement Contentions at 1-2 (**emphasis added**).

Sarepta further claims that "[a] physician or healthcare provider administering NS's Viltepso® (viltolarsen) product in accordance with its approved labeling will directly infringe, either literally or under the doctrine of equivalents, each asserted claim of the Wilton Patents, in violation of 35 U.S.C. § 271(a)." *Id.* at 2.

Sarepta further claims that "NS actively induces and intentionally encourages, aids, and abets the manufacture, offer for sale, sale, import, and use of Viltepso . . . in violation of 35 U.S.C. § 271(b)." *Id.*

Sarepta further claims that "NS contributes to, and will continue to contribute to, the direct infringement of the asserted claims of the Wilton Patents . . . in violation of 35 U.S.C. § 271(c)." *Id.*

Sarepta further claims that "NS's infringement is also willful." *Id.* at 3.

Accordingly, Sarepta's infringement contentions assert that NS directly, indirectly, and willfully infringes each of claims 1 and 2 of the '851 Patent, claims 1 and 2 of the '590 Patent, and claims 1 and 2 of the '827 Patent.

III. NS's Noninfringement Contentions

For the reasons set forth in detail below, Sarepta's infringement contentions are without merit. NS reserves the right to supplement or amend these contentions as discovery in this case proceeds.

A. Sarepta Has Failed to Meet Its Burden to Demonstrate Infringement By A Preponderance of the Evidence

It is Sarepta's burden to prove that NS infringes each and every asserted claim of the UWA Patents by a preponderance of the evidence. *Imhaeuser v. Buerk*, 101 U.S. 647, 662 (1879) ("[T]he burden to prove infringement never shifts if the charge is denied in the plea or answer."); *Accord Jazz Photo Corp. v. Int'l Trade Comm'n.*, 264 F.3d 1094, 1102 (Fed. Cir. 2001) ("The initial burden is upon the complainant to establish its cause of action, here patent infringement; the patentee must present evidence sufficient to establish that one or more patent claims are infringed."); *Nutrinova Nutrition Specialities & Food Ingredients GmbH v. Int'l. Trade Comm'n.*, 224 F.3d 1356, 1359 (Fed. Cir. 2000) ("As a general proposition, the law places the burden of

proving infringement on the patentee who alleges it."); *Centricut, L.L.C. v. Esab Group, Inc.*, 390 F.3d 1361, 1367 (Fed. Cir. 2004) ("The patentee has the burden of proving infringement by a preponderance of the evidence."); *Rohm & Haas Co. v. Brotech Corp.*, 127 F.3d 1089, 1092 (Fed. Cir. 1997) ("Infringement requires proof by a preponderance of the evidence."); *SRI Int'l. v. Matsushita Elec. Corp. of Am.*, 775 F.2d 1107, 1123 (Fed. Cir. 1985) ("The patentee bears the burden of proving infringement by a preponderance of the evidence.").

Sarepta has identified its evidence alleging that NS directly, indirectly, and willfully infringes each of claims 1 and 2 of the '851 Patent, claims 1 and 2 of the '590 Patent, and claims 1 and 2 of the '827 Patent. However, this evidence in insufficient to demonstrate that NS directly, indirectly, or willfully infringes any of the Asserted Claims. Accordingly, it is NS's contention that Sarepta has failed to meet its burden to demonstrate infringement of any of the Asserted Claims by a preponderance of the evidence.

NS may rely upon Sarepta's admissions made in pleadings, responses to interrogatories and requests for admission, as further evidence that Sarepta has failed to meet its burden to demonstrate infringement of any of the Asserted Claims by a preponderance of the evidence. NS likewise notes that depositions are ongoing, and that it may rely upon the testimony of Sarepta's, UWA's, Nippon Shinyaku's, and NS Pharma's witnesses as yet further evidence that Sarepta has failed to meet its burden to demonstrate infringement of any of the Asserted Claims by a preponderance of the evidence. NS further states that it may rely on expert reports and expert testimony of both Sarepta and NS experts as further evidence that Sarepta has failed to meet its burden to demonstrate infringement of any of the Asserted Claims by a preponderance of the evidence. NS reserves the right to supplement or amend these contentions as discovery in this case proceeds.

B. NS Does Not Infringe Any Valid Asserted Claim Because Each Asserted Claim of the UWA Patents is Invalid

As set forth in NS's Invalidity Contentions, served on July 11, 2023, each of the Asserted Claims of the UWA Patents is invalid. Accordingly, NS does not and cannot infringe any valid claim of the UWA Patents. As set forth in NS's Invalidity Contentions, NS may rely upon Sarepta's admissions made in pleadings, responses to interrogatories and requests for admission, as evidence that NS does not and cannot infringe any valid claim of the UWA Patents. NS likewise notes that depositions are ongoing, and that it may rely upon the testimony of Sarepta's, UWA's, Nippon Shinyaku's, and NS Pharma's witnesses as yet further evidence that NS does not and cannot infringe any valid claim of the UWA Patents. NS further states that it may rely on expert reports and expert testimony of both Sarepta and NS experts as further evidence that NS does not and cannot infringe any valid claim of the UWA Patents. NS reserves the right to supplement or amend these contentions as discovery in this case proceeds.

C. The Manufacture, Use, Sale, Offer for Sale, or Importation of Viltepso Does Not Infringe Any Asserted Claim Because the Base Sequence of Viltepso Does Not Comprise at Least 12 Consecutive Bases of SEQ ID NO: 195

Each of claims 1 and 2 of the '851 Patent, claims 1 and 2 of the '590 Patent, and claims 1 and 2[1] of the '827 Patent require an "antisense oligonucleotide . . . comprising a base sequence" and **"wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195)."** (emphasis added).

Sarepta has claimed that "[b]y making, using, selling, offering to sell, and/or importing Viltepso® (viltolarsen), NS **directly and indirectly** infringes the asserted claims of the Wilton Patents [i.e., the UWA patents]." Final Infringement Contentions at 1-2 (**emphasis added**).

[1] Claim 2 does not expressly recite this language, but claim 2 depends from claim 1, which does recite the language.

However, the sequence of bases in Viltepso is "CCTCCGGTTC TGAAGGTGTT C." *See e.g.,* NS00035784 at -790; NS00035807 at -813; NS00067092 at -098; NS's Responses and Objections to Sarepta's First Set of Request for Admission at 8 ("Nippon Shinyaku and NS Pharma further admit that the structure and base sequence of viltolarsen are shown in Figure 1," which shows the base sequence "CCTCCGGTTC TGAAGGTGTT C."). Accordingly, the sequence of bases in Viltepso contains only thymine bases and no uracil bases. *Id.*; *see also* NS's Responses and Objections to Sarepta's First Set of Request for Admission at 9 ("Nippon Shinyaku and NS Pharma further admit that the base sequence of viltolarsen contains thymine bases and does not contain uracil bases."). On the other hand, each possible set of at least 12 consecutive bases of "CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195)" contains at least one uracil base. Therefore, the manufacture, use, sale, offer for sale, or importation of Viltepso does not infringe any asserted claim because the base sequence of Viltepso does not comprise at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195).

Sarepta attempts to confuse the issue by improperly grouping the limitation "wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195)" with a separate claim limitation "in which uracil bases are thymine bases." *See* Final Infringement Contentions at Exhibits A, B and C. Sarepta groups the limitations together in an attempt to suggest that the phrase "in which uracil bases are thymine bases" modifies the preceding limitation "wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195)." *Id.* at Exhibit A p. 18 ("The nucleotide base sequence of Viltepso® (viltolarsen) is 'CCTCCGGTTC TGAAGGTGTT C,' see, e.g., Viltepso® Label 2021, NS00035784-794 at -790, **which comprises 12 consecutive bases of**

'CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195),' in which uracil bases are thymine bases." (emphasis added)).

Yet, Sarepta argued and the Court confirmed that the phrase "in which uracil bases are thymine bases" does not modify the preceding phrase, but modifies the antisense oligonucleotide as a whole. *See* Claim Construction Memorandum Opinion, DI 248 at 25-29. In analyzing the term "in which uracil bases are thymine bases," the Court first pointed to the grammatical structure of the claim language:

> The plain language of the claim uses commas to set off each "wherein" and "in which" clause, suggesting that **the "in which uracil bases are thymine bases" term modifies the antecedent subject "an antisense oligonucleotide."** To be sure, the claim also uses serial commas before every "wherein" and "in which" clause, which generally suggest, as a matter of grammar, a listing of individual, independent items in a series. . . . Moreover, **if the "in which uracil bases are thymine bases" term modified the sequence of bases immediately preceding it, i.e., SEQ ID NO: 195, as NS contends, then there would be no reason the patentee used a comma to separate the "in which uracil bases are thymine bases" term from the rest of the claim language**.

Id. at 26-27. In addition, the Court pointed to the prosecution history as evidence that "the applicant identified **'uracil bases [being] thymine bases' as a feature of the claimed antisense oligonucleotide rather than a feature of the preceding 'base sequence' limitation**." *Id.* at 27-28.

In view of the entirety of the evidence, the Court concluded that "each listed element, including 'uracil bases are thymine bases,' **independently modifies** the antecedent subject 'an antisense oligonucleotide' as a whole rather than a feature of the preceding 'base sequence' limitation." *Id.* at 28. In doing so, the Court adopted Sarepta's proposed construction that it argued for throughout the claim construction process. Sarepta cannot argue for a different construction now.

The result of the Court's construction is that the claimed "antisense oligonucleotide" must meet two "independent" limitations:

- The antisense oligonucleotide must include a base sequence "wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195)."
- "[T]he antisense oligonucleotide has thymine bases instead of uracil bases." DI 249 at 2.

Since Viltepso does not include a base sequence "wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195)" NS does not literally infringe any claim of the UWA Patents, whether directly, indirectly or willfully.

While Sarepta has included general allegations that NS may infringe under the doctrine of equivalents, Sarepta has not provided any substantive contentions related to any allegations under the doctrine of equivalents. *See e.g.,* Final Infringement Contentions at Exhibits A, B and C. Sarepta's failure to provide any substantive contentions related to infringement under the doctrine of equivalents demonstrates that Sarepta cannot meet its burden to prove infringement as a matter of law.

Nonetheless, any contention that NS infringes under the doctrine of equivalents cannot succeed due to the dedication-disclosure doctrine. "When a patentee discloses subject matter but does not claim it, the patentee dedicates the unclaimed subject matter to the public and cannot recapture it through the doctrine of equivalents." *Indivior Inc. v. Dr. Reddy's Labs., S.A.,* 930 F.3d 1325, 1346 (Fed. Cir. 2019) (citing *Johnson & Johnston Assoc. v. R.E. Serv. Co.,* 285 F.3d 1046, 1054 (Fed. Cir. 2002) (en banc)). To determine whether the disclosure-dedication doctrine applies in a given case, the court asks whether the specification discloses unclaimed subject matter with "such specificity that one of ordinary skill in the art could identify the subject matter that had been

disclosed and not claimed." *Eagle Pharms. Inc. v. Slayback Pharma LLC,* 958 F.3d 1171, 1175 (Fed. Cir. 2020) (quoting *PSC Comput. Prods., Inc. v. Foxconn Int'l, Inc.,* 355 F.3d 1353, 1360 (Fed. Cir. 2004)). "If the court concludes that the inventor dedicated an alleged equivalent to the public, the patent owner cannot prevail on its doctrine of equivalents infringement claim based on that equivalent." *Eagle Pharms*., 958 F.3d at 1175.

Here, any alleged equivalent has been disclosed in the patent, dedicated to the public, and cannot be the basis of a claim under the doctrine of equivalents. Here, the alleged equivalent limitation would require an antisense oligonucleotide comprising a base sequence "wherein the base sequence comprises at least 12 consecutive bases of CUG AAG GUG UUC UUG UAC UUC AUC C (SEQ ID NO: 195)" except wherein the uracil bases of SEQ ID NO: 195 are replaced with thymine bases instead. However, this "equivalent" is expressly disclosed in the specification of the UWA Patents at Table 1A:

TABLE 1A-continued

Description of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA. Since these 2'-O-methyl antisense oligonucleotides are more RNA- like, U represents uracil. With other antisense chemistries such as peptide nucleic acids or morpholinos, these U bases may be shown as "T".

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5'-3')
192	H53A(+39+62)	CUG UUG CCU CCG GUU CUG AAG GUG
193	H53A(+39+69)	CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G
194	H53D(+14-07)	UAC UAA CCU UGG UUU CUG UGA
195	H53A(+23+47)	CUG AAG GUG UUC UUG UAC UUC AUC C

See e.g., '851 Patent at Col. 19 (reciting SEQ ID NO: 195 with uracil bases, but explaining that "these U[racil] bases may be shown as "T[hymine]" bases instead). Since any alleged infringing

"equivalent" has been dedicated to the public, NS cannot infringe any claim of the UWA Patents under the doctrine of equivalents.

In support of its positions, NS may rely upon Sarepta's admissions made in pleadings, responses to interrogatories and requests for admission, as evidence that NS does not infringe the UWA Patents because Viltepso does not include at least 12 consecutive bases of SEQ ID NO: 195 either literally or under the doctrine of equivalents. NS likewise notes that depositions are ongoing, and that it may rely upon the testimony of Sarepta's, UWA's, Nippon Shinyaku's, and NS Pharma's witnesses as yet further evidence that NS does not infringe the UWA Patents because Viltepso does not include at least 12 consecutive bases of SEQ ID NO: 195 either literally or under the doctrine of equivalents. NS further states that it may rely on expert reports and expert testimony of both Sarepta and NS experts as further evidence that NS does not infringe the UWA Patents because Viltepso does not include at least 12 consecutive bases of SEQ ID NO: 195 either literally or under the doctrine of equivalents. NS reserves the right to supplement or amend these contentions as discovery in this case proceeds.

D. NS Does Not Directly Infringe Claims 1 and 2 of the '827 Patent Because It Does Not Administer an Antisense Oligonucleotide to a Patient

Claims 1 and 2[2] of the '827 Patent require "administering to the patient an antisense oligonucleotide." Sarepta has claimed that "[b]y making, using, selling, offering to sell, and/or importing Viltepso® (viltolarsen), NS **directly** and indirectly infringes the asserted claims of the Wilton Patents [i.e., the UWA patents]." Final Infringement Contentions at 1-2 (**emphasis added**). However, in setting forth its specific evidence in its claim charts Sarepta has not identified substantive contentions that NS directly infringes claims 1 and 2 of the '827 Patent by

[2] Claim 2 does not expressly recite this language, but claim 2 depends from claim 1, which does recite the language.

further evidence that NS does not willfully infringe the UWA Patents. NS further states that it may rely on expert reports and expert testimony of both Sarepta and NS experts as further evidence that NS does not willfully infringe the UWA Patents. NS reserves the right to supplement or amend these contentions as discovery in this case proceeds.

Dated: July 11, 2023

MORGAN, LEWIS & BOCKIUS

/s/Amy M. Dudash
Amy M. Dudash (DE Bar No. 5741)
1201 N. Market Street
Suite 2201
Wilmington, Delaware 19801
Telephone: 302.574.3000
Fax: 302.574.3001
amy.dudash@morganlewis.com

Attorneys for Plaintiff/Counterclaim Defendant Nippon Shinyaku Co., Ltd. and Counterclaim Defendant NS Pharma, Inc.

Amanda S. Williamson (admitted *pro hac vice*)
Christopher J. Betti (admitted *pro hac* vice)
Krista V. Venegas (admitted *pro hac* vice)
Wan-Shon Lo (admitted *pro hac* vice)
Maria E. Doukas (admitted *pro hac vice*)
Zachary Miller (admitted *pro hac vice*)
Guylaine Haché (admitted *pro hac vice*)
Michael T. Sikora (admitted *pro hac vice*)
110 N. Wacker Drive, Ste 2800
Chicago, IL 60601
Telephone: 312.324.1000
Fax: 312.324.1001
amanda.williamson@morganlewis.com
christopher.betti@morganlewis.com
krista.venegas@morganlewis.com
shon.lo@morganlewis.com
maria.doukas@morganlewis.com
zachary.miller@morganlewis.com
guylaine.hache@morganlewis.com
michael.sikora@morganlewis.com

Eric Kraeutler (admitted *pro hac vice*)
Alison Patitucci (admitted *pro hac vice*)
1701 Market Street
Philadelphia, PA 19103
Telephone: 215.693.5000
Fax: 215.963.5001
eric.kraeutler@morganlewis.com
alison.patitucci@morganlewis.com

EXHIBIT 13

REDACTED

IN ITS

ENTIRETY